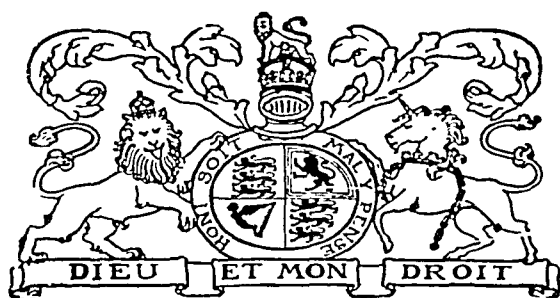


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SOME OBSERVATIONS ON VOGES-PROSKAUER TEST.*

BY

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[Received for publication, May 24, 1943.]

THE fact that the original Voges-Proskauer (V. P.) test often gives faint and indefinite reactions has led to the elaboration of several modifications by different workers. The most useful of these is that described by Barritt (1936). It consists in the addition of alpha-naphthol before the addition of the caustic alkali to the culture in glucose-phosphate medium and this results in an intensification of the colour change. The sensitiveness of the test is thus increased without loss of specificity. The purpose of the present work is to study some of the factors on which the test depends and to find out the optimum conditions for doing the test. Both V. P. and Barritt tests were studied.

Although the British Ministry of Health (1941) recommends the sterilization of the medium used in the V. P. test at 10-pound pressure, in our experience sterilization in a steamer at 100°C. on three successive days proved more satisfactory. This was adopted in these experiments.

Period of incubation.—One of the drawbacks of the V. P. test is that the period of incubation originally recommended is three days and it takes another 24 hours after the addition of the reagent for reading the result. There is also evidence that the long incubation increases the liability of positives becoming negatives (Paine, 1927; Tittsler, 1938). In a series of tests done by the writer on known *aerogenes* strains, 71 per cent of 139 strains gave negative results after incubation for 3 to 4 days. In the present work 20 known *aerogenes* strains were grown in glucose-peptone broth (bactopeptone) at 37°C. and tested after 1, 2, 3 and 4 days' incubation. The standard glucose-phosphate medium was used as control. The results are given in Table I :—

TABLE I.

		DAYS OF CULTURE.							
		1 day.		2 days.		3 days.		4 days.	
		+	—	+	—	+	—	+	—
V. P. test	Glucose-phosphate medium ..	18	2	19	1	8	12	8	12
	Glucose-peptone medium ..	18	2	19	1	19	1	19	1
Barritt test	Glucose-phosphate medium ..	20	0	20	0	15	5	9	11
	Glucose-peptone medium ..	20	0	20	0	20	0	20	0

It will be noted that with both the V. P. and the Barritt tests better results were obtained when the medium contained no phosphate. The results were the same after 1, 2, 3 or 4 days' incubation with this medium. But in a medium containing phosphate, positives tended to become negative after more than two days' incubation.

Phosphate in the medium.—The fact that a larger number of positives can be obtained when the medium contains no phosphates and that the presence of phosphate in the medium turns positives into negatives if incubation is prolonged, led to a study of the rôle of phosphates

* Paper read at the 31st Session of the Indian Science Congress held at Delhi on 3rd January, 1944.

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Peptone used in the medium.—It has been found that the use of different brands of peptone gives different results on known strains (*vide* Table IV).

TABLE IV.

	Bactopeptone.			Institute peptone.			Witte peptone.			Stearn's peptone.		
	+	±	—	+	±	—	+	±	—	+	±	—
V. P. with glucose-phosphate medium.	8	1	11	9	2	9	14	3	3	18	1	1
V. P. with glucose-peptone medium.	11	4	5	12	1	7	16	1	3	18	2	0
Barritt with glucose-phosphate medium.	13	7	0	15	5	0	20	0	0	20	0	0
Barritt with glucose-peptone medium.	20	0	0	20	0	0	20	0	0	20	0	0

Variation in results with different brands of peptone may be due to the presence in varying amounts of a substance containing guanidine grouping. This is known to react with diacetyl to produce a pink colour. It may also be due to the defect of the phosphates present in peptone. Different brands of peptone tested in this Institute have been found to contain different amounts of phosphate. Owing to the high sensitiveness of the Barritt test, the presence of small amounts of phosphate in the peptone does not affect its reliability. But this does not hold true for the V. P. test.

pH of the medium.—The effect of variation of pH of the medium on V. P. and Barritt tests are shown below. The tests were done with 3-day cultures:—

TABLE V.

BARRITT TEST.							
Glucose-phosphate medium.				Glucose-peptone medium.			
	+	±	—	+	±	—	
pH 7	10	3	7	20	0	0	
pH 7.5	9	0	11	20	0	0	
pH 8	9	0	11	20	0	0	

V. P. TEST.							
	+	±	—				
pH 7	8	0	12	16	1	3	
pH 7.5	8	0	12	15	4	1	
pH 8	8	0	12	19	0	1	

It will be seen from Table V that in glucose-phosphate medium there is no difference in the results of V. P. and Barritt tests on varying the pH. But in glucose-peptone medium pH 8 gives much better results than lower pH in the V. P. test, while in the Barritt test variation in pH had no effect.

DISCUSSION.

Positive V. P. test is due to some intermediary products of glucose fermentation (acetoin). V. P. positive organisms as shown by Tittsler may not stop at producing acetoin from

the epidemic described above an opportunity was offered to study the incidence of the serological types of the vibrios isolated and this report deals with the results obtained from the investigation.

ISOLATION.

Most of the cholera patients were seen at the Isolation Hospital in Kunming. Since our laboratories are located in the country we adopted a rectal-swabbing technique for collecting specimens. Cotton-wool swabs were prepared and sterilized in test-tubes. Each tube before using was filled with 10 c.c. of peptone water of the following composition:—

Potassium nitrite	1 g.
Sodium chloride	100 g.
Peptone	100 g.
Distilled water, add up to	10,000 c.c.
(Reaction adjusted to pH 9.0)				

Dry, or wetted with peptone solution, the swab after loading was placed in a tube of peptone water and taken to the laboratory in a special metal box. Upon arrival, it was immediately incubated at 37°C. At the end of six hours one alkaline agar plate and a fresh tube of peptone water were inoculated with the surface fluid. The plate was examined after incubating for 12 hours. If suspicious colonies were found, four of them were routinely transferred to agar slants, and if negative, two additional plates were streaked with the surface growth from the peptone-water sub-culture. Specimens negative on two platings were considered negative. Altogether 129 rectal specimens were collected from 110 cases of clinical cholera (1 case swabbed thrice; 17 cases, twice; 92 cases, once) and from them 83 strains of vibrio (including two inagglutinable strains) were obtained. Incidentally, stools from 12 normal persons, 8 cases of diarrhoea or dysentery, 1 case with coma of unknown cause were also cultured, but all were negative. Five specimens of water collected from wells in infected areas were also examined and from two of them two strains of inagglutinable vibrios were isolated. The results of examination are given in the first three columns of Table I:—

TABLE I.

Biochemical and serological study of the isolated vibrios.

Strain.	Date of isolation.	Origin.	Characters.	Hæmolytic test.	SERUM AGGLUTINATION.		
					Stock.	SERUM. Inaba.	Ogawa.
1	12-5-42	Case	Typical	—	Micro	640	..
2	12-5-42	"	"	—	"	X	X
3	12-5-42	"	"	—	"	640	..
4	15-5-42	"	"	—	"	2560	..
5	15-5-42	"	"	—	"	1280	80
6	15-5-42	"	"	—	"	1280	..
7	26-5-42	"	*Typical	—	"	1280	..
8	6-6-42	"	Typical	—	"	640	80
9	6-6-42	"	"	—	"	640	..
10	6-6-42	"	"	—	"	640	40
11	6-6-42	"	"	—	"	1280	40
12	6-6-42	"	"	—	"	2560	40
13	6-6-42	"	"	—	"	1280	40
14	6-6-42	"	"	—	"	640	40
15	6-6-42	"	"	—	"	1280	80
16	10-6-42	"	"	—	"	X	X
17	10-6-42	"	"	—	"	1280	40

ENHANCEMENT OF DIPHTHERIA TOXIN PRODUCTION BY GLYCOLYSIS.

BY

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AND

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[Received for publication, November 22, 1943.]

MUELLER (1938) observed with culture of *C. diphtheriae* in liquid medium containing commercial lactic acid (d and l forms) that after removal of an initial crop of zinc dl-lactate an abundant crop of zinc l-lactate was subsequently obtained from the filtrate. From this he concluded that utilization of lactic acid by *C. diphtheriae* was pretty definitely limited to the d-form, and the greater the amount of l-form present, the less was available for the growth of the organism. Sodium and ammonium salts of lactic acid have been successfully used by various workers for enhancement of toxin production (Pope and Lingwood, 1939; Pappenheimer *et al.*, 1937). From these observations it was expected that if the lactate be present as salt of the d-acid, more potent toxin might be obtained and an investigation on this line was undertaken.

EXPERIMENTAL.

It has been shown by one of us (Sen, 1943) that sarcolactic acid (d-lactic acid) may be produced in veal infusion by the glycolytic breakdown of glucose. This process was utilized in preparing a medium rich in sarcolactic acid.

Preparation of medium (A).—To 1 kg. freshly slaughtered veal, 0.05 M sodium phosphate (Na_2HPO_4) 500 c.c.; 4 per cent sodium bicarbonate solution 200 c.c.; glucose 17 g. and distilled water 1,300 c.c. were added and the whole was incubated at 30°C. to 32°C. for 12 hours with occasional shaking. No attempt was made to make the process anaerobic as it has been shown by Mayerhof (1930) that glycolytic process in muscle extract was not inhibited by the presence of oxygen. After 12 hours, the infusion was diluted further with one litre of water and the whole was placed in a water-bath at 80°C. and maintained at this temperature for $\frac{1}{2}$ hour. The pH of the infusion at this stage was usually found to be between 5.5 and 6.0 due to the lactic acid formed. A sample was kept for the estimation of lactic acid according to the method of Friedemann and Graeser (1933) and the pH of the whole substrate was adjusted at 8.2 with 40 per cent caustic soda solution. Ten c.c. of pancreatic extract (Cole and Onslow) were added every $\frac{1}{2}$ hour for 6 hours and temperature was maintained at 50°C. Thirty c.c. glacial acetic acid were next added and the whole was boiled for 30 minutes, filtered through lint and kept in the ice-chest overnight. Final dilution with water was then made so that the ultimate concentration of sarcolactic acid in the medium was 250 mg. to 260 mg. per 100 c.c. of the digest, pH of the broth was adjusted to 8.0, filtered through paper, 0.3 per cent maltose was added and the medium was sterilized by steaming on 3 consecutive days. On the average, a dilution of 4 parts of water for 1 part veal was found suitable but in some lots a higher percentage of sarcolactic acid was obtained and a 1 in 6 dilution was used.

For comparative studies, the medium (B), subsequently described, was used side by side with the above medium.

Preparation of medium (B).—To 1 kg. freshly slaughtered veal usually from the same lot as used for the medium (A), 3,000 c.c. water were added and the whole placed in the

TABLE I—concl'd.

Strain.	Date of isolation.	Origin.	Characters.	Hæmolytic test.	SERUM AGGLUTINATION.		
					SERUM.		
					Stock.	Inaba.	Ogawa.
80	2-8-42	Case	*Typical	—	1280	160	..
81	2-8-42	"	"	—	610	..	640
82	..	"	"	+	Inagglutinable		
83	..	"	"	+	"		
84	..	Water	"	+	"		
85	..	"	"	+	"		
El Tor	..	C. R. I., Kasauli	"	+	1280	X	X
Java	..	"	"	+	1280	X	X
Inaba	..	"	"	—	610	X	X
Ogawa	..	"	"	—	1280	X	X

'Typical' means Gram-negative, actively motile, comma vibrio which gave positive cholera-red reaction and liquefied gelatine; '*Typical', fermented glucose, maltose, mannite and saccharose with acid production in addition to other characters; Micro, microscopical agglutination with a stock serum dilution 1: 20 to 1: 100; figures, highest dilution of serum in which a positive reaction occurred; X, test not done.

CULTURAL STUDIES.

Young agar slant cultures obtained from plates as described above were studied microscopically for motility, serum agglutination (against a stock serum), as well as other characters such as morphology and staining reaction (Gram). When suggestive, representative cultures were chosen, one for each specimen, and kept in Hitchen's semi-solid medium for further study. The process of identification then proceeded with biochemical investigations including gelatine liquefaction, cholera-red reaction, carbohydrate fermentation and their effect on red blood corpuscles (Greig's technique). The results are given in the fourth and fifth columns of Table I.

SEROLOGICAL INVESTIGATION.

As stated previously it would be of great interest to see whether the different serological types of the cholera vibrio could be identified among the strains isolated. One strain each of Inaba and Ogawa organisms were obtained from the Central Research Institute, Kasauli, India. Both of them were in the smooth phase in so far as they formed typical smooth colonies and caused no spontaneous agglutination in saline. The Inaba or 'original' strain showed amongst vibrio bodies some long and spiral forms and on the whole the motility was not active, while the Ogawa or 'variant' form was typical in morphology and actively motile. They were strongly agglutinated by our stock cholera serum (Inaba to 1 : 2560 and Ogawa to 1 : 1280). Both strains appeared to be highly toxic, for in the course of immunization several rabbits succumbed to comparatively small doses of the boiled antigens. With these standard strains we prepared the specific sera in the following fashion: A 24-hour growth of the strain on agar slants was washed off in normal saline and the suspension boiled for two hours in a water-bath. This destroys the non-specific thermolabile 'H' antigen while the specific thermostable 'O' antigen is left intact. This suspension was diluted to contain approximately 10 billion organisms per cubic centimetre. Several rabbits were immunized using a two-week schedule.

First week .. Monday, 0.5 c.c. subcutaneously.
Wednesday, 1 c.c. „ .
Rest for 4 days.

Second week .. Monday, 0·5 c.c. intravenously.
Wednesday, 1 c.c. „
Rest for 4 days, then trial bleeding.

ACKNOWLEDGMENT.

Our thanks are due to Mr. Diptish Chakravarty, B.Pharm., for his useful technical assistance.

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From Table II it will be seen that, with the exception of two strains (276, 284), all the other old cultures belong to Inaba type. Whether strains 276 and 284 belong to the 'intermediate' type of Nobechi, as they were agglutinated by both of the sera equally well, has not been ascertained. It is curious that the old stock cultures were less easily agglutinated than the fresh strains. Their titre was comparatively low and sometimes the agglutination was only partial and indistinct.

CLINICAL STUDY.

Cholera is an acute disease. In most of our cases the clinical symptoms lasted from three to seven days. Exceptionally brief or protracted cases did occur but were comparatively rare. Before collecting the specimen for bacteriological examination the case history was carefully scrutinized particularly in regard to the day of illness, the stage of the disease, the severity of the infection and whether or not the patient had been prophylactically inoculated. This was done for the purpose of correlating the bacteriological findings with the clinical condition and the stage of the attack. The date of illness begins with the onset of diarrhoea and vomiting. The clinical stages are defined as acute, late and convalescent. The acute stage comprises the ordinary three clinical stages, namely preliminary diarrhoea, collapse and period of reaction; the late stage denotes a time when the patient still has some diarrhoea but the frequency has diminished and the stool becomes atypical; convalescent patients are free from diarrhoea. In judging the severity of the infection we took the frequency of bowel movements as an index. Cases which started with more than 30 in the first 24 hours are considered as severe, those with 20 to 30 as moderate, and those less than 10 as light. Some of these divisions are very artificial and may even be illogical but they serve roughly for the purpose. The findings are given in Table III:—

TABLE III.

Clinical conditions in relation to recovery of vibrio.

DAYS OF ILLNESS.			
Day of illness.	Number of specimens.	Positive cultures.	Negative cultures.
1	9	9	..
2	24	19	5
3	22	17	5
4	12	7	5
5	6	5	1
6	11	4	7
7	10	8	2
8	5	1	4
9	1	..	1
10	4	..	4
11	1	..	1
12	1	1	..
13
14	2	..	2
CLINICAL STAGE.			
Clinical stage.	Number of specimens.	Positive cultures.	Negative cultures.
Acute ..	54	44	10
Late ..	29	19	10
Convalescent ..	16	4	12

SOME PHYSICAL AND IMMUNOLOGICAL DIFFERENCES BETWEEN NATURAL AND RE-CONSTITUTED HUMAN SERUM.

BY

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[Received for publication, December 27, 1943.]

A COMPARISON has been made between natural and re-constituted human serum with respect to the following items: colour, pH, viscosity, anticomplementary titre, hæmagglutination titre and reaction with antihuman serum.

The samples of natural serum examined were obtained from the Red Cross Blood Bank, Calcutta. These represented samples of pooled and filtered serum prepared for subsequent drying from a frozen state. The interval which had elapsed between drawing of the blood and drying of the serum varied in different samples from 7 to 12 days.

The samples of re-constituted sera examined were prepared by the addition of distilled water to dried powders. In addition, another form of re-constituted serum, viz. that obtained by saline extraction of dried stains, was included in the tests to determine the reactions with antihuman serum. The latter type of re-constituted serum has previously been reported to be defective (Grevall, Chandra and Bhattacharji, 1943).

Care was taken to ensure the equality of concentration of the dilution of natural and re-constituted sera. In the case of the serum re-constituted from powder, distilled water was added to the powder to make up exactly to the original volume of the natural serum. The froth index was identical in both the natural and the re-constituted sera. The equality of concentration of serum protein in the saline extracts from stain was determined by the froth index only.

Hæmagglutination titre was determined by the method described previously (Grevall, Chandra and Woodhead, 1941). Open preparations consisting of mixtures of equal volumes of sera and suspensions of red blood corpuscles A or B were made on glass slides which were allowed to remain in a moist chamber for 30 minutes. The titre recorded was the *initial* dilution, in accordance with the serological usage (in bacteriological usage the titre is taken from the *ultimate* dilution).

The results obtained are recorded in the Table. Samples bearing the same roman numeral were derived from the same original serum pool.

The following conclusions and suggestions emerge:—

1. *Viscosity.*—Re-constituted serum is slightly more viscous and definitely more opaque than natural serum, yet the froth index of the two sera is the same.
2. *pH.*—Drying from a frozen state and re-constitution do not alter the pH.
3. *Precipitin reaction.*—The precipitin reaction of re-constituted serum is slightly lowered.

sub-type has been discussed and the significance of the two serological types given. No 'intermediate' type was recovered from the epidemic but in going over some of the old stock cultures, two suggestive strains have been found.

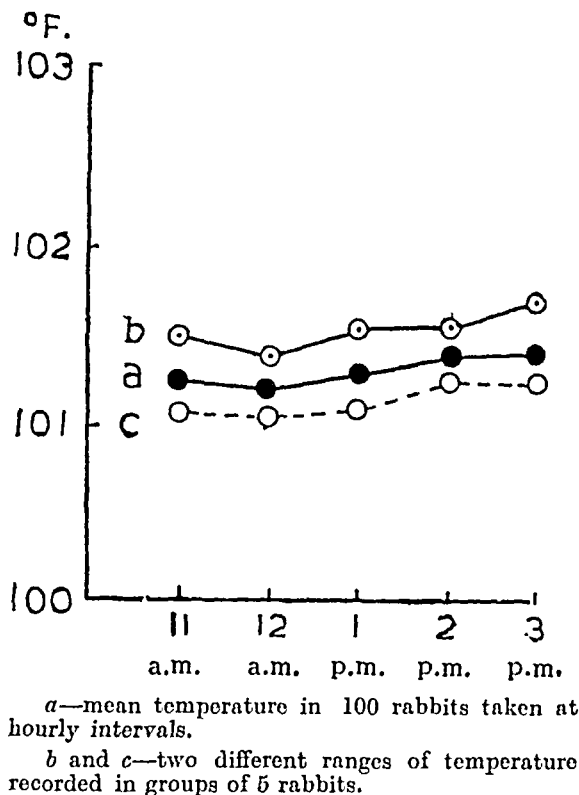
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The distilled-water samples were freshly collected, 0.85 per cent pure NaCl was added and the samples were then autoclaved at 120°C. for one hour. The reservoir water, prior to similar treatment, was incubated for 24 hours at 37°C. and kept for 48 hours at room temperature. Each sample of water was tested on one or more groups of 5 animals observing the

GRAPH 1.

Normal temperature curve.



precautions previously described, and using an equal number of untreated rabbits as controls. Animals were distributed according to sex and weight in each group. In every case one or two initial temperature records were made before the injection of the water and 3 to 4 subsequent to it. The fluid was injected intravenously at body temperature at the rate of 5 c.c. per minute. The total dose injected was calculated on the basis of 1 c.c. per 100 g. body-weight of the animal. The results are recorded in Table I:—

TABLE I.

Effect of intravenous injection of various samples of water on the rectal temperature of groups of 5 rabbits.

Serial number of sample.	ORDINARY DISTILLED WATER.		DOUBLE-DISTILLED WATER.		RESERVOIR WATER.	
	Mean temperature variation in injected rabbits, °F.	Mean temperature variation in control rabbits, °F.	Mean temperature variation in injected rabbits, °F.	Mean temperature variation in control rabbits, °F.	Mean temperature variation in injected rabbits, °F.	Mean temperature variation in control rabbits, °F.
1	0.4	0.3	0.3	0.5	2.1	0.3
2	0.9	0.5	0.8	0.5	1.9	0.4
3	1.2	0.3	1.1	0.3	1.7	0.3
4	0.4	0.3	0.5	0.3
5	0.9	0.5	0.5	0.5

DAILY MEASUREMENTS OF BASAL METABOLISM, BODY TEMPERATURE AND PULSE RATE DURING A JOURNEY TO THE TROPICS*.

BY

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[Received for publication, September 27, 1943.]

It has been demonstrated (Mason, 1934, 1940) that a change of residence from temperate to tropical climate produces in some individuals a fall of approximately 10 per cent in the basal metabolic rate. This was true of thirteen, or 62 per cent, of twenty-one English and American women studied in two climates. In several of these cases measurements had been made immediately before sailing from London or New York and again immediately after arrival in Madras, and it was found that the change had taken place during the journey and that the lower rate of heat production was established by the time of arrival.

With the purpose of finding out how long an exposure to tropical climate was necessary before the change became established, E. D. M., a woman aged 42, height 169 cm., weight 54 kg., who was known to be one in whom this adaptation occurred, took a Benedict-Roth metabolism apparatus with her on her journey from San Francisco to Madras in June 1940. She was travelling alone but was exceedingly fortunate in finding at once a fellow-passenger who was a nurse and had had experience in making metabolism measurements, and before the nurse disembarked at Yokohama another fellow-passenger going all the way to India was trained in the technique of measurement. During the week's halt at Manila between boats she stayed in the nurses' bungalow adjoining St. Luke's Hospital and with her own apparatus set up in her room was measured by the nurse who regularly did metabolism tests for that hospital†. Thus, it was possible to get daily tests on one subject before rising in the morning, all through the journey which involved an abrupt transition from comfortable temperate climate to the tropics. The only exceptions to the daily tests were from San Francisco to Honolulu, when the oxygen cylinder could not be located on the boat, and one day, 9th June, when the subject spent the night on land in Tokyo and travelled overland to rejoin the boat at Kobe. The voyage ended at Colombo, but control measurements were made in Madras on the same apparatus immediately after arrival there.

The tests, graphically recorded, were satisfactory in all cases. The voyage was smooth and the subject was not sea-sick. Diet was regular and kept approximately uniform in quantity and quality. Deck space was very limited in the class in which she was travelling, so the voyage was of necessity inactive, and accompanied by a slight gain in weight, but the inactivity was uniform throughout the period of experiment. Thus, the only changing factor was climate.

The results are shown in the Table and charted in the Graph. The climatic change in sailing south from Kobe to Manila occurred at approximately latitude 22°N. and was ideally abrupt for such a study. Up to 11th June it was cool, from 12th June on it was thoroughly tropical. The charted daily changes in basal metabolism show on the first and second days after entering the tropics a higher metabolic rate, accompanied by a slightly higher body temperature. The metabolism began to fall from the third day, and by 19th June, one

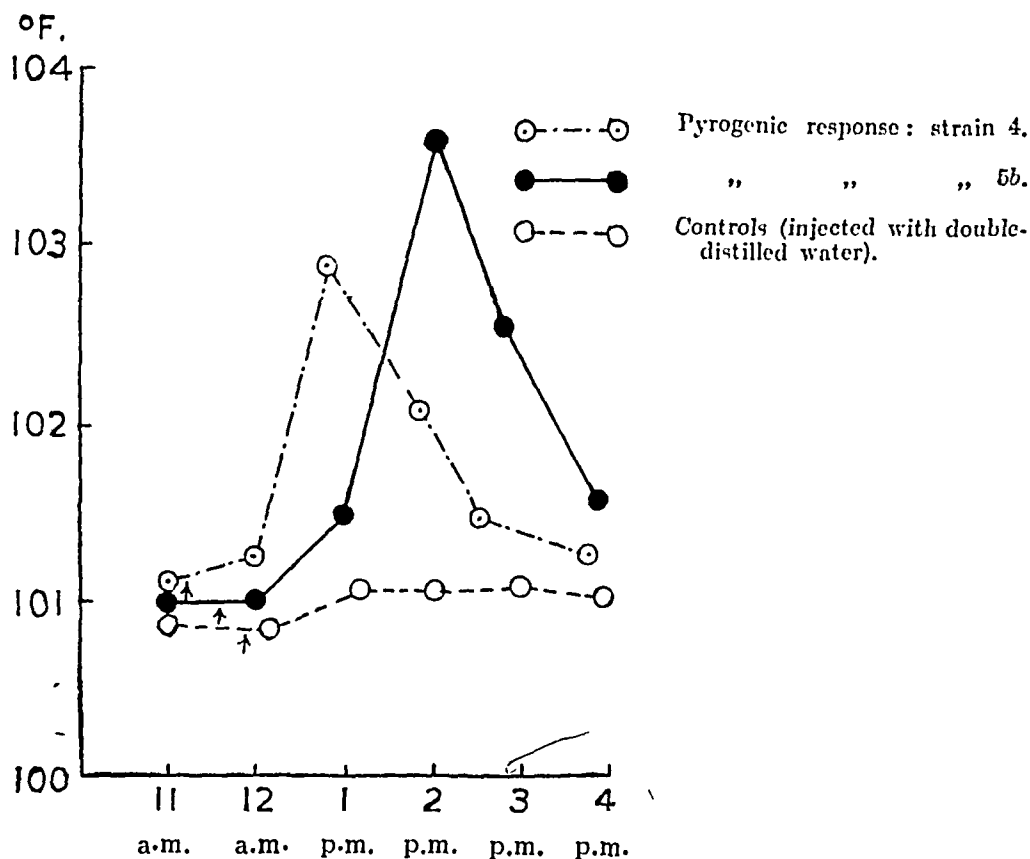
* The writer gratefully acknowledges the interest of Dr. Cecil B. Drinker of the Department of Physiology, Harvard School of Public Health, in encouraging her to undertake this experiment, and the gift of the Benedict-Roth metabolism apparatus and other accessories from Harvard University.

† The writer is under much obligation to these three women who rose early every morning to make the measurements: Miss R. Engler, B.N., Mrs. Gwendolyn Torkelson and Mrs. Charlotte Mallory Howell, B.N., of St. Luke's Hospital, Manila.

series of rabbits in parallel with the pyrogenic test in order to determine which was best suited to routine laboratory work. Eleven rabbits were subjected to temperature recordings and leucocyte estimations at regular intervals for one week prior to injection with pyrogenic substance. Temperature was recorded immediately before taking blood samples to avoid any influence on temperature of the procedures of blood collection. On

GRAPH 2.

Pyrogenic response from strains 4 and 5b. The arrows indicate the time of injection of the fluids containing the cultures. Average of 5 rabbits in each case.



completion of this period of observation, a suspension of each of strains 4 and 5b in double-distilled water representing 25 million organisms per cubic centimetre was injected in doses of 1 c.c./100 g. body-weight. Leucocyte counts were made using Neubauer type of haemocytometer and hourly rectal temperatures were recorded. The results are shown in Table III.

Suspensions of strains 4 and 5b produced definite and marked hyperpyrexia in all of the 6 rabbits injected with each strain. They also produced a slight fall in the leucocyte counts amounting on the average to 1,783 and 2,341 per c.mm. respectively for the same two groups of animals. The average hourly variation in leucocyte counts in these rabbits prior to injection was about 1,000 per cubic millimetre. Thus the reduction of leucocytes following injection of pyrogenic fluid could not be considered significant in view of the possibility of errors involved in any blood-counting technique. In our experiments a reduction of the leucocyte count by 4,000 per cubic millimetre or over was never observed.

TABLE III.

Effect of Nos. 4 and 5b pyrogenic organisms on the total leucocyte counts and rectal temperatures of rabbits as compared with normal daily and weekly variations in the same animal.

NORMAL VARIATION.				VARIATION AFTER INJECTION OF FLUIDS.													
Serial number of rabbit.	Sex and weight, g.	Rectal temperature, F.	W.b.c. count per c.mm.	Rectal temperature, F.	W.b.c. count per c.mm.	Maximum daily variation.	Maximum weekly variation.	Fluids injected, dosage.	Rabbit number.	Initial temperature, F.	Initial w.b.c. count per c.mm.	W.b.c. count per c.mm. 1 hour after injection.	W.b.c. count per c.mm. 1½ hours after injection.	Rectal temperature 1 hour after injection, F.	Rectal temperature 3 hours after injection, F.	Temperature variations, F.	Variations in w.b.c. counts per c.mm.
1	♂ 1,520	0.3	750	0.6	1,650	Culture No. 4. 25 million/c.c. 1 c.c./100 g. weight of rabbit.	{		{	101.2	10,150	7,350	12,500	101.0	102.1	0.9	-2,600
2	♂ 1,557	0.1	1,200	0.2	2,050					100.8	8,050	4,150	4,500	100.6	102.2	1.4	-3,900
3	♂ 1,435	0.4	850	0.6	1,850					101.1	9,550	9,050	9,850	101.2	102.8	1.7	-500
4	♂ 1,470	0.2	1,200	0.5	1,500					101.5	7,100	6,500	8,000	102.1	103.4	1.9	-600
5	♂ 1,440	0.2	1,000	0.3	2,150					100.8	8,400	7,950	10,100	101.0	102.2	1.4	-450
																	-2,650
6	♂ 1,480	0.3	650	0.5	1,750	Culture No. 5b. 25 million/c.c. 1 c.c./100 g. weight of rabbit.	{		{	100.5	6,900	4,350	7,300	100.2	102.0	1.5	-2,550
7	♀ 1,280	0.2	950	0.2	1,000					101.2	8,550	6,800	6,000	101.1	102.8	1.6	-2,550
8	♂ 2,115	0.4	300	0.5	950					101.3	8,050	4,550	5,050	103.4	104.5	3.2	-3,500
																	-2,900
																	-2,100
																	-2,100
																	-2,450
9	♂ 2,000	0.2	800	0.2	1,050	Double-distilled water control. 1 c.c./100 g. weight of rabbit.	{		{	101.1	6,050	6,500	7,000	101.4	101.0	0.3	+950
10	♀ 2,350	0.4	950	0.4	950					101.0	12,400	10,800	11,650	101.1	101.5	0.5	-1,600
11	♂ 1,750	0.5	850	0.6	1,700					101.3	10,850	9,350	10,600	101.7	101.6	0.4	-1,500
																	+350

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SOME OBSERVATIONS ON VOGES-PROSKAUER TEST.*

BY

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THE fact that the original Voges-Proskauer (V. P.) test often gives faint and indefinite reactions has led to the elaboration of several modifications by different workers. The most useful of these is that described by Barritt (1936). It consists in the addition of alpha-naphthol before the addition of the caustic alkali to the culture in glucose-phosphate medium and this results in an intensification of the colour change. The sensitiveness of the test is thus increased without loss of specificity. The purpose of the present work is to study some of the factors on which the test depends and to find out the optimum conditions for doing the test. Both V. P. and Barritt tests were studied.

Although the British Ministry of Health (1941) recommends the sterilization of the medium used in the V. P. test at 10-pound pressure, in our experience sterilization in a steamer at 100°C. on three successive days proved more satisfactory. This was adopted in these experiments.

Period of incubation.—One of the drawbacks of the V. P. test is that the period of incubation originally recommended is three days and it takes another 24 hours after the addition of the reagent for reading the result. There is also evidence that the long incubation increases the liability of positives becoming negatives (Paine, 1927; Tittsler, 1938). In a series of tests done by the writer on known *aerogenes* strains, 71 per cent of 139 strains gave negative results after incubation for 3 to 4 days. In the present work 20 known *aerogenes* strains were grown in glucose-peptone broth (bactopeptone) at 37°C. and tested after 1, 2, 3 and 4 days' incubation. The standard glucose-phosphate medium was used as control. The results are given in Table I:—

TABLE I.

			DAYS OF CULTURE.								
			1 day.		2 days.		3 days.		4 days.		
			+	—	+	—	+	—	+	—	
V. P. test	{	Glucose-phosphate medium	..	18	2	19	1	8	12	8	12
		Glucose-peptone medium	..	18	2	19	1	19	1	19	1
Barritt test	{	Glucose-phosphate medium	..	20	0	20	0	15	5	9	11
		Glucose-peptone medium	..	20	0	20	0	20	0	20	0

It will be noted that with both the V. P. and the Barritt tests better results were obtained when the medium contained no phosphate. The results were the same after 1, 2, 3 or 4 days' incubation with this medium. But in a medium containing phosphate, positives tended to become negative after more than two days' incubation.

Phosphate in the medium.—The fact that a larger number of positives can be obtained when the medium contains no phosphates and that the presence of phosphate in the medium turns positives into negatives if incubation is prolonged, led to a study of the rôle of phosphates

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in the V. P. test. Harden and Young (1905) had found that the addition of inorganic phosphate increases sugar fermentation by yeast. But similar results have not been recorded for bacteria. Hence experiments were conducted in which V. P. and Barritt tests were done after culturing *aerogenes* strains in glucose medium containing varying amounts of phosphate. The results are given in Tables II and III :—

TABLE II.

		V. P. TEST.									BARRITT TEST.					
		24 hours.			48 hours.			72 hours.			24 hours.			48 hours.		
		+	±	—	+	±	—	+	±	—	++	+	++	+	++	+
Glucose-peptone	20	0	0	20	0	0	18	1	1	20	0	20	0	20	0
No phosphate	20	0	0	20	0	0	19	1	0	20	0	20	0	20	0
Glucose-peptone	20	0	0	20	0	0	18	2	0	20	0	20	0	19	1
Phosphate 0·1 per cent	..	20	0	0	20	0	0	15	1	4	20	0	20	0	17	3
Glucose-peptone	20	0	0	15	5	0	8	7	5	20	0	20	0	15	5
Phosphate 0·2 per cent	..	20	0	0	10	8	2	5	6	9	20	0	19	1	15	5
Glucose-peptone	20	0	0	10	8	2	5	6	9	20	0	19	1	15	5
Phosphate 0·3 per cent	..	20	0	0	10	8	2	5	6	9	20	0	19	1	15	5
Glucose-peptone	20	0	0	10	8	2	5	6	9	20	0	19	1	15	5
Phosphate 0·4 per cent	..	20	0	0	10	8	2	5	6	9	20	0	19	1	15	5
Glucose-peptone	20	0	0	10	8	2	5	6	9	20	0	19	1	15	5
Phosphate 0·5 per cent	..	20	0	0	10	8	2	5	6	9	20	0	19	1	15	5

TABLE III.

		Number of <i>aerogenes</i> strains.		Positive.	Negative.
				Per cent.	Per cent.
V. P. test with medium containing phosphate	..	139	40 (28·78)	99 (71·22)	
V. P. test with medium containing no phosphate	..	174	154 (88·51)	20 (11·49)	
Barritt test with medium containing phosphate	..	124	100 (80·65)	24 (19·35)	
Barritt test with medium containing no phosphate	..	124	120 (96·78)	4 (3·22)	

In Table III the results are after 3 to 4 days' incubation.

It will be seen that when the Barritt test is done with 24- or 48-hour cultures there is no material variation in the result even if the phosphate content of the medium is varied. But when the same test was done on 72-hour cultures the number of negatives increased when the phosphate content was increased.

If the V. P. test is done with a 24-hour culture there is no change in the number of positives. But if 48- or 72-hour cultures are used the presence of phosphates decreases the number of positives.

A possible explanation is that the phosphate enhances the fermentation of glucose and leads to a further breakdown of the acetoin if the incubation is prolonged.

Peptone used in the medium.—It has been found that the use of different brands of peptone gives different results on known strains (*vide* Table IV).

TABLE IV.

	Bactopeptone.			Institute peptone.			Witte peptone.			Stearn's peptone.		
	+	±	—	+	±	—	+	±	—	+	±	—
V. P. with glucose-phosphate medium.	8	1	11	9	2	9	14	3	3	18	1	1
V. P. with glucose-peptone medium.	11	4	5	12	1	7	16	1	3	18	2	0
Barritt with glucose-phosphate medium.	13	7	0	15	5	0	20	0	0	20	0	0
Barritt with glucose-peptone medium.	20	0	0	20	0	0	20	0	0	20	0	0

Variation in results with different brands of peptone may be due to the presence in varying amounts of a substance containing guanidine grouping. This is known to react with diacetyl to produce a pink colour. It may also be due to the defect of the phosphates present in peptone. Different brands of peptone tested in this Institute have been found to contain different amounts of phosphate. Owing to the high sensitiveness of the Barritt test, the presence of small amounts of phosphate in the peptone does not affect its reliability. But this does not hold true for the V. P. test.

pH of the medium.—The effect of variation of pH of the medium on V. P. and Barritt tests are shown below. The tests were done with 3-day cultures:—

TABLE V.

BARRITT TEST.							
Glucose-phosphate medium.				Glucose-peptone medium.			
	+	±	—		+	±	—
pH 7	10	3	7	20	0	0	0
pH 7.5	9	0	11	20	0	0	0
pH 8	9	0	11	20	0	0	0

V. P. TEST.							
	+	±	—		+	±	—
pH 7	8	0	12	16	1	3	3
pH 7.5	8	0	12	15	4	1	1
pH 8	8	0	12	19	0	1	1

It will be seen from Table V that in glucose-phosphate medium there is no difference in the results of V. P. and Barritt tests on varying the pH. But in glucose-peptone medium pH 8 gives much better results than lower pH in the V. P. test, while in the Barritt test variation in pH had no effect.

DISCUSSION.

Positive V. P. test is due to some intermediary products of glucose fermentation (acetoin). V. P. positive organisms as shown by Tittsler may not stop at producing acetoin from

glucose. If conditions are favourable the acetoin is likely to be further broken down and utilized by the organism as a source of carbon and thus a V. P. positive organism may become V. P. negative.

To get the best results, the test should be done under conditions which will ensure the presence of acetoin. With many *aerogenes* strains prolonged incubation at 37°C. in a medium containing phosphate may break down the acetoin. In order to prevent this a shorter period of incubation is desirable. This is probably the reason why in the latest edition of the British Ministry of Health's Report No. 71 (1941) incubation for two days has been recommended in place of the usual three days. One hundred per cent of known strains give positive results after 24 hours' incubation in a glucose-peptone medium. Our opinion is that the sensitivity of Barritt's test is definitely superior to the V. P. test and as such should be adopted.

Max Levine (1941) stresses the necessity for incubating the culture for five days as originally introduced by Clark and Lubs and the temperature recommended is 30°C. It appears that this low temperature merely helps in slowing the metabolism of the organism and preventing the breaking down of acetoin. But this method besides being a dilatory one is also disadvantageous because, in most laboratories, incubators are kept at 37°C. and not at 30°C. It may be argued, however, that in identifying a *coliform* organism not only V. P. but also Methyl Red (M. R.) tests are usually done. As the M. R. test is done after 3 days it would be advantageous to do both tests at the same time. In such a case we would recommend the Barritt test to be done with glucose-peptone medium and the M. R. test with glucose-phosphate medium.

CONCLUSION.

Even under optimum conditions the original Voges-Proskauer test is never so satisfactory as the Barritt test and the latter is best done after two days' or even one day's incubation at 37 C. in glucose-phosphate or glucose-peptone medium. If it is desired to do the V. P. or Barritt test after the usual three days' incubation, instead of after one day's incubation, glucose-peptone of pH 8 should be used in place of glucose-phosphate medium.

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ENHANCEMENT OF DIPHTHERIA TOXIN PRODUCTION BY GLYCOLYSIS.

BY

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MUELLER (1938) observed with culture of *C. diphtheriae* in liquid medium containing commercial lactic acid (d and l forms) that after removal of an initial crop of zinc dl-lactate an abundant crop of zinc l-lactate was subsequently obtained from the filtrate. From this he concluded that utilization of lactic acid by *C. diphtheriae* was pretty definitely limited to the d-form, and the greater the amount of l-form present, the less was available for the growth of the organism. Sodium and ammonium salts of lactic acid have been successfully used by various workers for enhancement of toxin production (Pope and Lingwood, 1939; Pappenheimer *et al.*, 1937). From these observations it was expected that if the lactate be present as salt of the d-acid, more potent toxin might be obtained and an investigation on this line was undertaken.

EXPERIMENTAL.

It has been shown by one of us (Sen, 1943) that sarcolactic acid (d-lactic acid) may be produced in veal infusion by the glycolytic breakdown of glucose. This process was utilized in preparing a medium rich in sarcolactic acid.

Preparation of medium (A).—To 1 kg. freshly slaughtered veal, 0.05 M sodium phosphate (Na_2HPO_4) 500 c.c.; 4 per cent sodium bicarbonate solution 200 c.c.; glucose 17 g. and distilled water 1,300 c.c. were added and the whole was incubated at 30°C. to 32°C. for 12 hours with occasional shaking. No attempt was made to make the process anaerobic as it has been shown by Mayerhof (1930) that glycolytic process in muscle extract was not inhibited by the presence of oxygen. After 12 hours, the infusion was diluted further with one litre of water and the whole was placed in a water-bath at 80°C. and maintained at this temperature for $\frac{1}{2}$ hour. The pH of the infusion at this stage was usually found to be between 5.5 and 6.0 due to the lactic acid formed. A sample was kept for the estimation of lactic acid according to the method of Friedemann and Graesser (1933) and the pH of the whole substrate was adjusted at 8.2 with 40 per cent caustic soda solution. Ten c.c. of pancreatic extract (Cole and Onslow) were added every $\frac{1}{2}$ hour for 6 hours and temperature was maintained at 50°C. Thirty c.c. glacial acetic acid were next added and the whole was boiled for 30 minutes, filtered through lint and kept in the ice-chest overnight. Final dilution with water was then made so that the ultimate concentration of sarcolactic acid in the medium was 250 mg. to 260 mg. per 100 c.c. of the digest, pH of the broth was adjusted to 8.0, filtered through paper, 0.3 per cent maltose was added and the medium was sterilized by steaming on 3 consecutive days. On the average, a dilution of 4 parts of water for 1 part veal was found suitable but in some lots a higher percentage of sarcolactic acid was obtained and a 1 in 6 dilution was used.

For comparative studies, the medium (B), subsequently described, was used side by side with the above medium.

Preparation of medium (B).—To 1 kg. freshly slaughtered veal usually from the same lot as used for the medium (A), 3,000 c.c. water were added and the whole placed in the

water-bath at 80°C. Digestion and subsequent processes were carried out just as in medium (A).

Production of toxin.—Broth was left at room temperature for at least 48 hours before inoculation. The Park-Williams 8 strain was used throughout the experiment. The cultures were incubated at 35°C. and toxin harvested after 7 days. Dermonecrotic test was done on guinea-pigs and the number of Lf doses present per cubic centimetre was determined. Results are recorded in Table I:—

TABLE I.

Medium.	Number of experiments.	AVERAGE RESULTS.			
		Total nitrogen mg. per 100 c.c. broth.	Sarcolactic mg. per 100 c.c. broth.	Lf doses per c.c. of toxin.	MRD per c.c. of toxin.
(A)	32	350	250 to 260	39	25×10^4
(B)	34	330	125 to 140	27	15×10^4

Maximum flocculation titre of toxin in medium (A) was 54 Lf doses per c.c. and the minimum figure was 26. Corresponding figures for medium (B) were 40 and 16. With 1 in 6 dilution of broth in medium (A) keeping sarcolactic acid concentration approximately at 250 to 260 mg. per 100 c.c. and with 1 in 6 dilution of medium (B), similar differences as shown in Table I were observed.

Statistical evaluation of results (Burn, 1937):—

TABLE II.

Medium.	Number of experiments.	Mean result \pm S. E.	T.
(A)	32	39 ± 1.7	} 5.7
(B)	34	27 ± 1.21	

where, S. E. = Standard error,

T. = Test of significance,

$$= \frac{m_A - m_B}{\sqrt{e_A^2 + e_B^2}},$$

m_A and m_B are respective mean results and e_A and e_B respective standard errors of media (A) and (B).

SUMMARY.

From the results given in the text it appears that there is significant enhancement of diphtheria toxin production, using a veal infusion medium prepared in the manner described which favours the glycolytic production of sarcolactic acid. This medium is inexpensive and can be prepared in any bacteriological laboratory. Observation over a prolonged period has shown that there is no marked seasonal variation in the result.

ACKNOWLEDGMENT.

Our thanks are due to Mr. Diptish Chakravarty, B.Pharm., for his useful technical assistance.

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SOME PHYSICAL AND IMMUNOLOGICAL DIFFERENCES BETWEEN NATURAL AND RE-CONSTITUTED HUMAN SERUM.

BY

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A COMPARISON has been made between natural and re-constituted human serum with respect to the following items: colour, pH, viscosity, anticomplementary titre, hæmagglutination titre and reaction with antihuman serum.

The samples of natural serum examined were obtained from the Red Cross Blood Bank, Calcutta. These represented samples of pooled and filtered serum prepared for subsequent drying from a frozen state. The interval which had elapsed between drawing of the blood and drying of the serum varied in different samples from 7 to 12 days.

The samples of re-constituted sera examined were prepared by the addition of distilled water to dried powders. In addition, another form of re-constituted serum, viz. that obtained by saline extraction of dried stains, was included in the tests to determine the reactions with antihuman serum. The latter type of re-constituted serum has previously been reported to be defective (Grevall, Chandra and Bhattacharji, 1943).

Care was taken to ensure the equality of concentration of the dilution of natural and re-constituted sera. In the case of the serum re-constituted from powder, distilled water was added to the powder to make up exactly to the original volume of the natural serum. The froth index was identical in both the natural and the re-constituted sera. The equality of concentration of serum protein in the saline extracts from stain was determined by the froth index only.

Hæmagglutination titre was determined by the method described previously (Grevall, Chandra and Woodhead, 1941). Open preparations consisting of mixtures of equal volumes of sera and suspensions of red blood corpuscles A or B were made on glass slides which were allowed to remain in a moist chamber for 30 minutes. The titre recorded was the *initial* dilution, in accordance with the serological usage (in bacteriological usage the titre is taken from the *ultimate* dilution).

The results obtained are recorded in the Table. Samples bearing the same roman numeral were derived from the same original serum pool.

The following conclusions and suggestions emerge:—

1. *Viscosity*.—Re-constituted serum is slightly more viscous and definitely more opaque than natural serum, yet the froth index of the two sera is the same.

2. *pH*.—Drying from a frozen state and re-constitution do not alter the pH.

3. *Precipitin reaction*.—The precipitin reaction of re-constituted serum is slightly lowered.

TABLE.

Showing reactions of three lots of pooled normal human serum in different phases.

Sera.	Colour.	Drops* in 1 c.c. from the same pipette (viscosity).	pH†	Anticomplementary titre‡ : haemolysis of sensitized r.b.c. with complement :—		Haemagglutination titre : 1 in :—						Formation of a rings with antihuman serum in dilutions of 1 in :—			
				1 MHD.	2 MHD.	1,000	10,000	20,000	40,000	60,000	80,000				
I, 1, Normal No. 197-2	.. Amber	23 plus a small vol.	8.1	$\frac{T}{T}$	$\frac{\pm}{\pm}$	4	+	+	+	+	+	+	+	+	+
I, 2, Re-constituted No. D42	.. Opalescent straw.	22 plus a small vol.	8.1	$\frac{T}{T}$	$\frac{?}{-}$	2	+	+	+	+	+	+	+	+	+
I, 3, Saline extract of dry stain of I, 1.	+	+	+	+	+	+	+	+	+
I, 4, Saline extract of dry stain of I, 2.	+	+	+	+	+	+	+	+	+
II, 1, Normal No. 198	.. Amber	22 plus a vol.	8.1	$\frac{+}{\pm}$	$\frac{?}{-}$	4	+	+	+	+	+	+	+	+	+
II, 2, Re-constituted No. D44	.. Opalescent straw.	22 plus a smaller vol.	8.1	$\frac{+}{T}$	$\frac{\pm}{?}$	2	+	+	+	+	+	+	+	+	+

The precipitin reaction of the saline extracts (= serum re-constituted from a dry state obtained without freezing) is markedly lowered. That this is not due to a deficiency of serum protein is established by the equality of the froth index with a known dilution of natural serum. The lowered reactivity results from a defective re-constitution alone. Extracts made with distilled water instead of saline solution are known to give even a poorer reaction.

The precipitin reaction of serum dried for the second time, in the ordinary way, as a stain, from re-constituted serum, is further lowered though slightly.

4. *Hæmagglutination titre.*—The hæmagglutination titre of pooled normal sera is much lower than the 1 in 16 titre recommended as a standard for the plasma of a safe universal donor (Greval and Chandra, 1939, 1941; Greval, Chandra and Woodhead, *loc. cit.*). Pooling besides weakening the sera a and b (from subjects B and A respectively) by dilution partly neutralizes the isonins (isohæmagglutinins) a and b with isogens (isohæmagglutinogens) A and B which are also present in the sera (B in a and A in b). Pooled serum is a weak serum ab with isonins a and b nearly equal. This equality of the isonins is not shown in the Table. The isolysins (Greval, Bhattacharji and Chowdhury, 1943), if present in some sera, are also neutralized or weakened by the same process. The titre of re-constituted serum is still lower.

5. *Anticomplementary titre.*—The anticomplementary titre of pooled natural serum and of re-constituted serum is definitely higher than that of most normal sera tested individually (as in the Wassermann reaction). This is significant in view of (i) the 'reactions, often severe, occurring when serum (not plasma S. D. S. G.) is employed intravenously' (Strumia, Wagner and Monaghan, 1940; Buttle, Kekwick and Schweitzer, 1940), and (ii) the fact that at least in the shock due to immunological causes (anaphylaxis) the decrease in the complement of the circulating blood is a finding (Topley, 1933). The questions arise (i) whether there is a decrease in the complement of the circulating blood in surgical shock also, (ii) whether a reduction (or a further reduction) of the complement and the consequent worsening of the clinical picture, after transfusion with human serum, have been responsible for the reported ill effects of human serum, (iii) whether the period of storage of sera collected for drying should be shortened (human serum is known to become anticomplementary on keeping), (iv) whether plasma, stored or dried, would be a better therapeutic agent than serum, and (v) whether the anticomplementary titre of the re-constituted sera (or of the stored liquid sera) should be reduced by heating them at 56°C. for half an hour (the 'inactivation' of the sera at 56°C. for half an hour in the complement fixation is done more to remove the anticomplementary agents than to render the native complement inactive).

ACKNOWLEDGMENT.

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DAILY MEASUREMENTS OF BASAL METABOLISM, BODY TEMPERATURE AND PULSE RATE DURING A JOURNEY TO THE TROPICS*.

BY

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It has been demonstrated (Mason, 1934, 1940) that a change of residence from temperate to tropical climate produces in some individuals a fall of approximately 10 per cent in the basal metabolic rate. This was true of thirteen, or 62 per cent, of twenty-one English and American women studied in two climates. In several of these cases measurements had been made immediately before sailing from London or New York and again immediately after arrival in Madras, and it was found that the change had taken place during the journey and that the lower rate of heat production was established by the time of arrival.

With the purpose of finding out how long an exposure to tropical climate was necessary before the change became established, E. D. M., a woman aged 42, height 169 cm., weight 54 kg., who was known to be one in whom this adaptation occurred, took a Benedict-Roth metabolism apparatus with her on her journey from San Francisco to Madras in June 1940. She was travelling alone but was exceedingly fortunate in finding at once a fellow-passenger who was a nurse and had had experience in making metabolism measurements, and before the nurse disembarked at Yokohama another fellow-passenger going all the way to India was trained in the technique of measurement. During the week's halt at Manila between boats she stayed in the nurses' bungalow adjoining St. Luke's Hospital and with her own apparatus set up in her room was measured by the nurse who regularly did metabolism tests for that hospital†. Thus, it was possible to get daily tests on one subject before rising in the morning, all through the journey which involved an abrupt transition from comfortable temperate climate to the tropics. The only exceptions to the daily tests were from San Francisco to Honolulu, when the oxygen cylinder could not be located on the boat, and one day, 9th June, when the subject spent the night on land in Tokyo and travelled overland to rejoin the boat at Kobe. The voyage ended at Colombo, but control measurements were made in Madras on the same apparatus immediately after arrival there.

The tests, graphically recorded, were satisfactory in all cases. The voyage was smooth and the subject was not sea-sick. Diet was regular and kept approximately uniform in quantity and quality. Deck space was very limited in the class in which she was travelling, so the voyage was of necessity inactive, and accompanied by a slight gain in weight, but the inactivity was uniform throughout the period of experiment. Thus, the only changing factor was climate.

The results are shown in the Table and charted in the Graph. The climatic change in sailing south from Kobe to Manila occurred at approximately latitude 22°N. and was ideally abrupt for such a study. Up to 11th June it was cool, from 12th June on it was thoroughly tropical. The charted daily changes in basal metabolism show on the first and second days after entering the tropics a higher metabolic rate, accompanied by a slightly higher body temperature. The metabolism began to fall from the third day, and by 19th June, one

* The writer gratefully acknowledges the interest of Dr. Cecil B. Drinker of the Department of Physiology, Harvard School of Public Health, in encouraging her to undertake this experiment, and the gift of the Benedict-Roth metabolism apparatus and other accessories from Harvard University.

† The writer is under much obligation to these three women who rose early every morning to make the measurements: Miss R. Engler, R.N., Mrs. Gwendolyn Torkelson and Mrs. Charlotte Mallory Howell, R.N., of St. Luke's Hospital, Manila.

week after entering the tropics, it had fallen to 28.1 calories per square metre per hour, 10 per cent less than the average rate for this subject in America. During the second week with fluctuations which may or may not have been related to the menstrual cycle, it continued to fall slightly more, the lowest record being 27.1 calories. By 26th June, two weeks after entering the tropics, it appears to have become established at the final tropical rate.

TABLE.

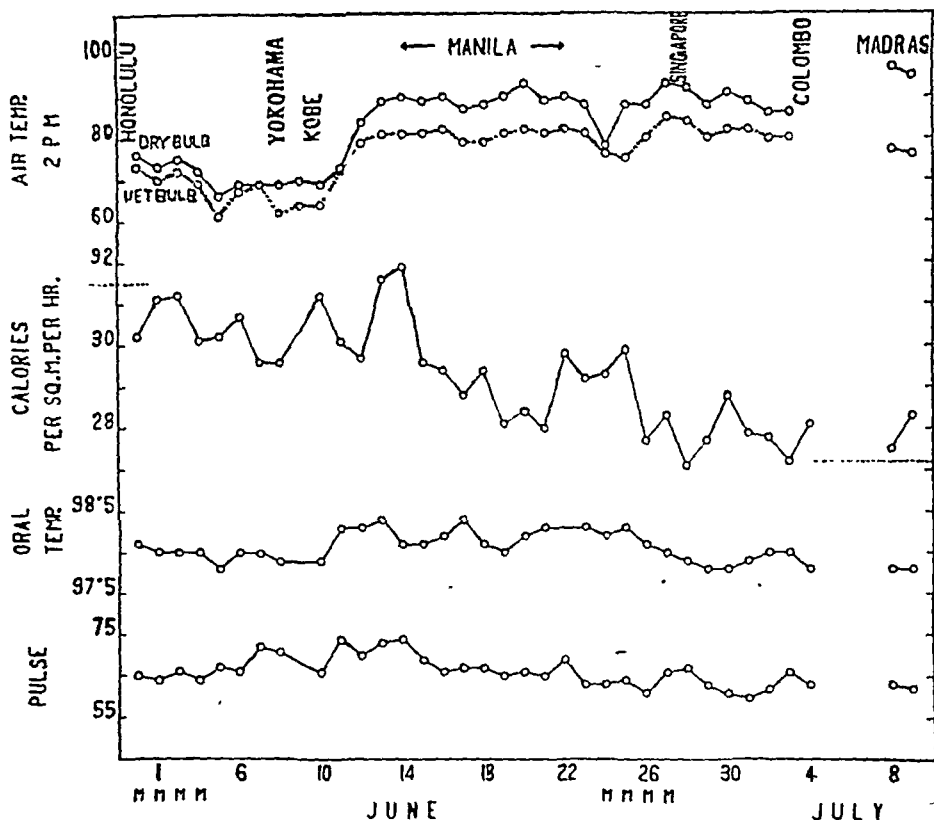
Daily basal measurements on E. D. M. of metabolism, mouth temperature and pulse rate on a journey from San Francisco to Madras.

Date.	Place.	Body-weight, g.	AIR TEMPERATURE, °F.		Mouth temperature, °F.	Pulse rate.	Calories square metre hour.	REMARKS.	
			Dry bulb.	Wet bulb.					
May 31	Leaving Honolulu	..	53.3	76	73	98.1	65	30.2	Menstruation.
June 1		73	70	98.0	64	31.1	
" 3		75	73	98.0	66	31.2	
" 4		72	69	98.0	64	30.1	
" 5		..	54.2	66	61	97.8	67	30.2	"
" 6		69	67	98.0	66	30.7	
" 7		..	54.2	69	69	98.0	72	29.6	
" 8	Nearing Yokohama	..	53.8	69	62	97.9	71	29.6	
" 9	Land Tokyo to Kobe	70	64	"
" 10	Leaving Kobe	..	54.0	69	64	97.9	66	31.2	
" 11		73	72	98.3	74	30.1	
" 12		..	54.4	84	79	98.3	70	29.7	
" 13		89	81	98.4	73	31.6	"
" 14	Manila harbour	90	81	98.1	74	31.9	
" 15	Manila land	89	81	98.1	69	29.6	
" 16	" "	90	82	98.2	66	29.4	
" 17	" "	87	79	98.4	67	28.8	"
" 18	" "	..	55.7	88	79	98.1	67	29.4	
" 19	" "	..	55.7	90	81	98.0	65	28.1	
" 20	" "	93	82	98.2	66	28.4	
" 21	" "	89	81	98.3	65	28.0	Measured at 5.30 a.m. on land.
" 22	Leaving Manila	90	82	(97.8)	69	29.8	
" 23		88	81	98.3	63	29.2	
" 24		78	76	98.2	63	29.3	
" 25		88	75	98.3	64	29.9	Menstruation.
" 26		88	80	98.1	61	27.7	"
" 27	Singapore harbour	93	85	98.0	66	28.3	"
" 28	" "	..	55.1	92	84	97.9	67	27.1	"
" 29	Nearing Penang	88	80	97.8	63	27.7	
" 30		91	82	97.8	61	28.8	
July 1		89	82	97.9	60	27.9	
" 2		86	80	98.0	62	27.8	"
" 3		86	80	98.0	66	27.3	
" 4	Colombo harbour	97.8	63	28.1	
" 5	Train	
" 6	Arrived Madras	After rising, in laboratory.
" 7		
" 8	Madras	..	53.9	97	77	97.8	63	27.5	
" 9	"	..	54.1	95	76	97.8	62	28.3	

This interpretation of the data, that while one week produced a marked change, another week was required for stabilization, is supported by the record of body temperatures. The chart shows that during the first two weeks in the tropics the body temperature was at its highest, but that after two weeks it had established itself once more at the normal level for all climates for this subject. This is in accord also with the data from an earlier voyage (Mason,

1940) when the mouth temperatures were found to be higher during the first ten days in the tropics and then to return to normal, and indicate that not until the body's mechanisms for the regulation of heat production have become adjusted to the new conditions does the body temperature return to its normal level.

GRAPH.



Daily basal measurements on E. D. M. of metabolism, mouth temperature and pulse rate on a journey from San Francisco to Madras.

(Air temperature taken from ship's records at 2 p.m. The fall in dry-bulb temperature on 24th June was due to a quickly passing rainstorm occurring at the time of measurement. At the edges of the chart are shown in dotted lines the average basal metabolic rate for this subject during the furlough year 1939-40 in America, at left, and during the first seven months after return to Madras, at right. Menstrual days are marked 'M'.
2nd June was dropped from the calendar on crossing the international meridian line.)

The basal pulse rate, highest at the first entrance into the tropics, decreased, with fluctuations, to a rate slightly slower than the average rate in temperate climate.

Since experiments of this kind are not likely to be recorded often it is important to compare closely these records with the only other previously recorded similar experiment known to the writer, that of Martin (1930). The two sets of data are strikingly consistent. In both the highest metabolic rate occurred on first entrance into the tropics, in both the fall occurred fairly rapidly, in his case during five days after reaching the tropics, in E. D. M.'s case during one week. In both the body temperature rose slightly simultaneously with the slight initial increase in metabolism. With the lowering of metabolism his body temperature also returned to normal, but his adjustment appears to have been completed within the first week.

COMPARISON OF TROPICAL STUDIES WITH LABORATORY STUDIES ON THE EFFECT OF ARTIFICIALLY REGULATED TEMPERATURE CHANGES.

Various workers have studied physiological reactions to artificially regulated changes in environmental temperature. Burton, Scott, McGlone and Bazett (1940) studying men living

for several days at different temperatures in an air-conditioned room found in the heat an initial rise in both body temperature and heat production followed by a return to normal temperature and a slow fall in heat production. Their data are consistent with the tropical data of Martin and of Mason, except that all the subjects so far studied (six) appear to have shown only one type of response, whereas Mason (1934, 1940) found two types among the 21 women studied in the tropics.

Hardy and Du Bois (1938) and Winslow, Herrington and Gagge (1937) working with men, unclothed, for short-period experiments of a few hours, found the metabolism approximately constant through temperatures ranging from 22°C. to 35°C. and from 6.5°C. to 35.5°C. respectively. But Hardy and Millhorat (1939) and Hardy and Du Bois (1940) found for women, studied under the same conditions as the men, a marked fall in heat production at temperatures above 27°C. They conclude that there is a difference between men and women in their response to warm environments. This conclusion is not supported by the tropical experiments of Martin and of Mason on themselves. Furthermore, the prompt fall in metabolism in response to heat reported for the women in these short-period experiments differs from the slow adaptation of several days in the two experimenters entering the tropics. In the latter condition, where the adjustment is relatively slower, the indication is that the diminution in heat production is a 'chemical regulation' and that it is the reduced activity of the thyroid gland which is the regulating factor*.

SUMMARY.

On a journey from San Francisco to India a woman whose basal metabolism was known to be 10 per cent lower in the tropics (Madras) than in a temperate climate (New York and Boston) was measured daily with the purpose of finding out how long an exposure to the tropics was necessary for this adaptation in heat production to be established. The measurements were made before rising in the morning, with a Benedict-Roth metabolism apparatus. The conditions throughout the journey were approximately uniform with respect to diet and activity and the transition from comfortably cool to hot humid tropical climate was abrupt.

On the first two days after exposure to tropical heat both the basal metabolism and oral temperature rose slightly. From the third day the metabolism began to fall and at the end of a week in the tropics was 10 per cent lower than the average rate for this subject in temperate climate. During the second week it fluctuated and fell slightly more. The oral temperature did not return to this subject's normal until the end of the second week and it is suggested that the second week was a period of stabilization of the heat balance.

These results are consistent with those of a similar experiment on himself reported by Martin, and support the hypothesis that the mechanism for this relatively slow adjustment of heat production on entering the tropics, beginning about the third day and not completed for approximately one week, is reduced activity of the thyroid gland.

A brief comparison is made of these tropical studies with laboratory studies by other workers on the effect of artificially regulated temperature changes.

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* There is an excellent discussion of regulation of temperature by varying heat production in Sir Charles J. Martin's paper.

STUDIES IN CALCIUM AND PHOSPHORUS METABOLISM.

Part VI.

THE IONIC PRODUCTS OF CALCIUM PHOSPHATES IN BLOOD SERUM OF RACHITIC INFANTS.

BY

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THE rôle of vitamin D in the prevention and cure of deficiency rickets is now well established. Its alleged influence on the absorption of calcium and phosphorus, either or both from the intestine, has been mainly held responsible for its curative and preventive action in rickets although the evidence on this question has been mainly inconclusive. Patwardhan and Chitre (1942) have reviewed such evidence and have shown that in albino rats the absorption from loops of intestine isolated *in situ*, proceeded equally well (a) in the state of vitamin D deficiency, (b) in cases where rats were protected from rickets by adequate dosage of vitamin D and (c) in induced hypervitaminosis D, early or late. It has not been possible, however, to find an adequate explanation for the fact that in absence of vitamin D the retention of calcium and/or phosphorus is adversely affected. Harris (1932) has observed that the retention of both these elements was improved by therapeutic doses of vitamin D, but again decreased when massive doses of the vitamin were administered.

Thus, it will appear that the mode of action of vitamin D so far as the retention of calcium and phosphorus is concerned still remains unclarified. A closer study of the development, progress and cure of rickets might have been expected to reveal the secret of vitamin D action. Yet intensive research work by numerous workers on various phases of rickets has failed to improve the situation. Apart from its action of increasing the retention of calcium and phosphorus, vitamin D brings about changes in the concentration of these elements in the blood and affects the deposition of calcium salts at the junction of epiphyseal cartilage and bone shaft in a young growing animal.

In 1919 Iversen and Lenstrup (quoted by Hess, 1930), and in 1921 Howland and Kramer pointed out that in rickets the serum inorganic phosphorus was decreased whereas the calcium remained more or less normal. Hess (*loc. cit.*) stated that in a number of infants when definite clinical signs of rickets were seen the calcium as well as the phosphorus level of the serum was normal. Elliot and Park (1942) observe that the decrease in calcium level of the serum during rickets is met with less frequently and even in such cases the lowering is well marked when tetany is co-existent. It is noteworthy that besides the study of serum calcium and phosphorus in rickets, other constituents of the serum as affected by rickets have received little attention.

Various workers have shown that the proteins of serum combine with a part of the serum calcium (Loeb, 1925; Loeb and Nichols, 1927; Marrack and Thacker, 1926). McLean and Hastings (1934, 1935) have applied the Mass Law Equation to deduce the relationship between

the total calcium, total protein and ionized calcium in serum. The calculated values of the ionized calcium agreed with those found by the frog-heart method developed by these authors. They observe that 'the calcium ion concentration of plasma at any one time is the resultant of an equilibrium between the total calcium and total protein present in the plasma. It is the ionic part of the calcium that is physiologically active and is kept within normal limits as far as possible. Fluctuations in protein level in response to total calcium are necessary to keep the $[Ca^{++}]$ within the narrow limits of normalcy'. Gutman and Gutman (1936, 1937) have made an observation that this relationship only holds good when the globulin content of the serum is normal. An increase in the concentration of this protein would require modification of the Mass Law Equation as applied by McLean and Hastings.

The other component of the serum which may influence the level of serum calcium is the inorganic phosphorus (Scholtz, 1931), which in its turn may depend upon the acid-base equilibrium.

Thus, it would appear that mere estimations of calcium and inorganic phosphorus in rachitic or normal serum would at best give an incomplete picture of the disturbances in metabolism reflected in the composition of the serum which provides the immediate internal environment of the site of bone deposition. It was felt therefore that a thorough study of many other constituents of serum besides calcium and inorganic phosphorus was desirable, if only to answer whether the composition of the protein mixture, the pH of the plasma, the total base, etc., were in any way influenced by the deficiency of vitamin D. The investigation, commenced four years ago, dealt with (1) clinical rickets observed in infants and children and (2) experimental rickets induced in dogs. This paper deals with the results of observations on children.

EXPERIMENTAL.

Infants and children of both sexes between the ages of 6 months to 5 years were examined by a clinician and in cases where rickets was frankly present or suspected to be present a blood sample by venepuncture was obtained. The blood was allowed to clot and the following determinations were carried out on the separated serum, (1) calcium, (2) inorganic phosphorus, (3) total protein and (4) albumin and globulin. The methods for the estimation of calcium and phosphorus have been described by Patwardhan and Chitre (1940) in an earlier paper. The total protein was estimated by Kjeldahl's method on 0.5 c.c. serum. The determination of albumin and globulin, total chlorides, total base, etc., was also carried out in most cases and the non-protein nitrogen in some cases: but since no result of any significance has been obtained they will not be further mentioned in this paper.

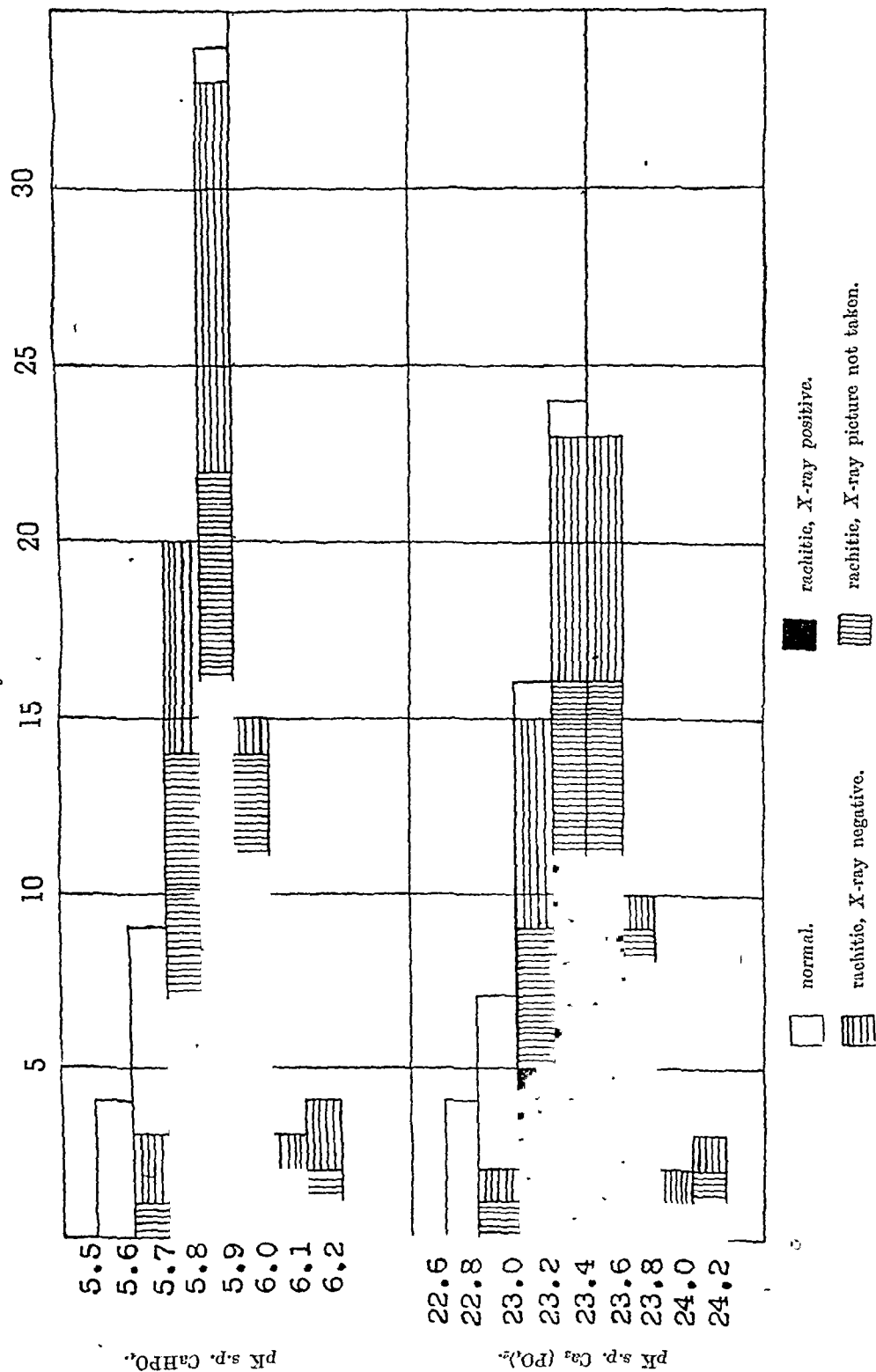
Blood serum from about one hundred cases has been analysed, but owing to difficulties which sometimes arise in obtaining an adequate amount of blood from the vein of infants complete data for 79 cases of suspected and frank rickets were available for study. The X-ray pictures of the distal ends of radius and ulna were taken in 54 of these cases, of which 44 showed characteristic radiological picture of rickets in varying degrees and 10 displayed doubtful or no rachitic changes in bone although the clinical diagnosis was that of rickets. In the remaining 25 no X-ray pictures were available, dependence being placed entirely on the diagnosis by the clinician. Unfortunately, the history and the clinical findings of most of the patients have been inadequately recorded and hence no correlation can be attempted between the clinical appraisal of the severity of rickets, the radiological evidence and the blood findings.

DISCUSSION.

The number of normal children whose blood serum has been analysed is only eleven. It is admittedly inadequate, yet certain differences between the rachitic and normal blood serum are markedly consistent and are in conformity with the findings in experimental rickets (to be published shortly).

GRAPH.

Number of cases.

Frequency distribution of the solubility products of CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$ in rachitic and normal children.

The Tables include all the relevant data. The values for $[Ca^{++}]$ in column 7 have been derived by reference to the nomogram of McLean and Hastings (1935a) based on the Mass Law Equation — $\frac{[Ca^{++}]}{[Ca \text{ proteinate}]} \times \frac{[\text{protein}^-]}{[\text{proteinate}^-]} = K$.

These authors have shown that the $[Ca^{++}]$ concentrations calculated thus agreed closely with those observed by the frog-heart method. Column 8 shows $[Ca^{++}]$ per kg. of serum water. By applying the considerations put forward and experimentally confirmed by Sendroy and Hastings (1926) the values for $[HPO_4^{=}]$ and $[PO_4^{\equiv}]$ per kg. of serum water have been calculated. In the last two columns are the values of pK s.p. $CaHPO_4$ and pK s.p. $Ca_3(PO_4)_2$. The pH used for calculating these values has been taken to be pH 7.40 for rachitic as well as non-rachitic infants. The study (to be published) of experimental rickets has shown that the pH of blood does not undergo change before the onset, during the development or on cure of rickets.

In the Graph the negative logarithms of the solubility products of $[Ca^{++}] \times [HPO_4^{=}]$ and $[Ca^{++}]^3 \times [PO_4^{\equiv}]^2$ are plotted for all the cases included in the Tables. No attempt has been made at the present moment to analyse these figures statistically but the frequency distribution of the solubility products of both these salts in rachitic and non-rachitic cases is extremely interesting. It will be convenient to consider and interpret these products separately for each salt.

Concentrations of calcium, inorganic phosphorus and proteins and the solubility products of $CaHPO_4$ and $Ca_3(PO_4)_2$ in the blood serum of normal and rachitic children.

TABLE A.

Rachitic children—radiologically positive.

Number.	Age. Years-months.	Sex.	PER 100 C.C. SERUM.			Ca ⁺⁺ , mg.	$[Ca^{++}] \times 10^{-3}$ M. per kg. H ₂ O.	$[HPO_4^{=}] \times 10^{-3}$ M. per kg. H ₂ O.	$[PO_4^{\equiv}] \times 10^{-8}$ M. per kg. H ₂ O.	pK s.p. $CaHPO_4$.	pK s.p. $Ca_3(PO_4)_2$.
			Calcium, mg.	Inorganic P, mg.	Total protein, g.						

Cases from Bai Jerbai Wadia Hospital for Children.

32	1-5	F	9.05	3.72	6.92	4.00	1.08	1.07	4.48	5.93	23.59
38	0-6	F	7.11	4.18	8.0	3.00	0.81	1.20	5.03	6.02	23.86
42	1-6	M	9.05	4.42	7.44	3.75	1.01	1.27	5.32	5.88	23.53
43	5-0	F	7.11	3.35	7.84	3.00	0.81	0.97	4.03	6.10	24.06
37	3-0	F	9.05	3.84	7.38	4.00	1.08	1.11	4.62	5.92	23.57
41	3-0	F	10.86	3.96	8.02	4.00	1.08	1.14	4.76	5.91	23.54
45	1-8	M	7.11	4.67	7.22	3.00	0.81	1.35	5.62	5.96	23.77
46	2-0	M	8.39	4.14	8.29	3.25	0.88	1.19	4.98	5.97	23.77
47	3-0	F	7.12	5.01	7.39	3.00	0.81	1.44	6.03	5.93	23.71
48	2-0	M	9.70	4.34	8.11	3.75	1.03	1.25	5.22	5.89	23.54
95	2-0	M	10.15	3.97	6.69	4.75	1.28	1.14	4.78	5.83	23.31

TABLE A—concl'd.

Number.	Age. Years—months.	Sex.	PER 100 C.C. SERUM.					$\left[\text{Ca}^{++} \right]$ kg. H ₂ O.	$\left[\text{HPO}_4^{=} \right]$ kg. H ₂ O.	$\left[\text{PO}_4^{=} \right]$ kg. H ₂ O.	pK s.p. CaHPO ₄ .	pK s.p. Ca ₃ (PO ₄) ₂ .
			Calcium, mg.	Inorganic P, mg.	Total protein, g.	Ca ⁺⁺ , mg.						
Cases from Bai Jerbai Wadia Hospital for Children—concl'd.												
65	10	M	8.08	4.40	8.09	3.50	0.94	1.27	5.30	5.91	23.62	
83	2-0	M	8.95	4.54	7.38	4.00	1.08	1.31	5.46	5.85	23.42	
84	2-0	F	10.54	4.43	7.38	4.50	1.21	1.28	5.33	5.81	23.28	
93	1-0	F	11.14	3.88	7.73	4.75	1.28	1.12	4.67	5.82	23.33	
96	10	M	10.94	4.76	6.81	5.00	1.35	1.37	5.73	5.73	23.09	
97	2-0	M	10.35	4.13	7.55	4.50	1.21	1.19	4.97	5.84	23.34	
80	1-0	M	8.49	4.03	6.30	4.00	1.08	1.16	4.85	5.90	23.53	
63	2-0	F	7.11	4.63	6.67	3.25	0.87	1.33	5.57	5.93	23.68	
68	1-6	F	8.44	3.47	6.49	3.75	1.01	1.00	4.18	5.99	23.74	
86	2-0	F	11.14	4.68	7.20	4.75	1.28	1.35	5.63	5.76	23.17	
90	2-8	F	11.34	4.54	7.25	4.00	1.08	1.31	5.46	5.84	23.51	
92	2-0	M	11.14	4.00	6.95	5.00	1.35	1.15	4.81	5.80	23.24	
87	2-9	F	10.54	4.42	7.33	4.50	1.21	1.27	5.32	5.81	23.29	
99	1-6	F	11.94	4.13	6.63	5.25	1.42	1.19	4.97	5.77	23.14	
78	2-6	F	7.96	4.10	6.75	3.50	0.94	1.18	4.93	5.95	23.68	
81	2-6	F	9.80	4.63	6.84	4.50	1.21	1.33	5.57	5.79	23.24	
89	1-0	M	11.14	4.06	6.90	5.00	1.35	1.71	4.88	5.80	23.23	
98	2-0	M	11.34	4.54	6.60	5.00	1.35	1.31	5.46	5.75	23.13	
100	0-7	F	9.75	4.27	7.22	4.25	1.15	1.23	5.14	5.85	23.40	

Cases from Byramjee Jeejeebhoy Hospital for Children.

1	1-6	F	10.35	4.37	7.01	4.50	1.21	1.26	5.26	5.81	23.30
2	0-4½	F	9.05	4.42	6.86	4.00	1.08	1.27	5.32	5.85	23.45
3	0-2½	M	10.98	4.16	5.23	5.80	1.56	1.20	5.01	5.72	23.01
4	0-5	M	9.24	5.43	5.66	4.50	1.21	1.56	6.53	5.71	23.11
5	1-6	M	9.69	3.80	6.05	4.75	1.28	1.09	4.57	5.85	23.35
6	0-5	M	9.79	4.21	6.23	4.50	1.21	1.21	5.06	5.82	23.33
7	0-4	M	9.70	4.88	6.96	4.25	1.15	1.41	5.87	5.79	23.28
8	0-6	F	10.35	3.84	7.27	4.50	1.21	1.11	4.62	5.86	23.41
9	2-0	M	9.69	2.66	6.32	4.50	1.21	0.76	3.20	6.02	23.73
10	1-0	F	9.69	3.57	6.34	4.50	1.21	1.03	4.30	5.89	23.47
11	1-0	F	8.39	4.13	6.85	3.75	1.01	1.19	4.97	5.91	23.59
12	—	—	9.69	4.63	6.25	4.50	1.21	1.33	5.57	5.78	23.24
13	0-8	M	7.75	5.00	5.51	3.75	1.03	1.44	6.02	5.83	23.42
14	0-8	M	10.98	5.00	6.14	5.25	1.42	1.44	6.02	5.69	22.98

TABLE B.

*Rachitic children—radiologically negative.**Cases from Bai Jerbai Wadia Hospital for Children.*

60	2-0	F	8.27	4.46	7.47	3.50	0.94	1.28	5.37	5.91	23.61
64	2-0	M	9.05	4.70	8.87	3.50	0.94	1.35	5.65	5.89	23.56
61	1-4	F	12.90	4.39	7.26	5.50	1.48	1.26	5.28	5.72	23.03
70	1-6	F	11.30	5.00	8.13	4.50	1.21	1.44	6.02	5.75	23.18
88	1-0	M	10.99	3.08	7.67	4.75	1.28	0.90	3.71	5.94	23.53
26	1-6	M	8.43	2.71	7.29	3.50	0.94	0.78	3.26	6.13	24.04
24	1-6	M	10.35	4.73	7.63	4.25	1.15	1.36	5.69	5.80	23.31
30	2-6	F	10.33	4.73	7.25	4.50	1.21	1.36	5.69	5.77	23.23
31	3-6	M	8.73	4.68	6.70	4.00	1.08	1.35	5.63	5.83	23.39
27	4-0	F	10.99	4.83	6.30	5.00	1.35	1.39	5.81	5.72	23.08

TABLE C.

Rachitic children—radiograms not taken.

Number.	Age. Years-months.	Sex.	Per 100 c.c. serum.		Total protein, g.	Ca ⁺⁺ , mg.	$\left[\text{Ca}^{++} \right] \times 10^{-3}$ M. per kg. H ₂ O.	$\left[\text{HPO}_4^{--} \right] \times 10^{-3}$ M. per kg. H ₂ O.	$\left[\text{PO}_4^{=} \right] \times 10^{-3}$ M. per kg. H ₂ O.	pK s.p. CaHPO ₄ .	pK s.p. Ca ₃ (PO ₄) ₂ .
			Calcium, mg.	Inorganic P, mg.							
72009	2-0	M	9.05	1.98	6.64	4.25	1.15	0.57	2.38	6.18	24.11
72276	5-0	M	9.70	2.20	6.83	4.25	1.15	0.63	2.65	6.13	23.97
71942	4-0	F	9.70	4.02	7.08	4.25	1.15	1.16	4.84	5.87	23.45
72324	1-6	M	10.09	6.39	7.51	4.25	1.15	1.81	7.69	5.67	23.05
72470	10	M	9.70	4.30	7.01	4.25	1.15	1.24	5.17	5.84	23.39
72546	3-0	M	10.99	3.93	7.26	4.75	1.28	1.14	4.73	5.83	23.32
72597	3-6	F	10.69	4.94	7.41	4.60	1.24	1.42	5.94	5.74	23.16
72737	0-8	M	10.86	6.16	7.45	4.75	1.28	1.77	7.41	5.64	22.93
69692	1-6	M	8.52	5.02	7.53	3.50	0.94	1.45	6.04	5.86	23.50
72945	3-0	M	10.99	4.38	8.04	4.50	1.21	1.26	5.27	5.81	23.29
72825	—	F	10.67	4.29	7.56	4.60	1.24	1.24	5.16	5.80	23.28
73024	2-9	F	9.70	4.24	6.60	4.25	1.15	1.22	5.10	5.85	23.40
73001	1-0	F	9.38	3.80	7.18	4.12	1.11	1.09	4.57	5.91	23.54
73110	4-0	M	11.64	2.49	6.89	5.12	1.38	0.72	2.99	6.00	23.62
73002	2-0	M	10.35	4.93	7.15	4.50	1.21	1.42	5.93	5.76	23.19
72685	2-0	M	10.35	4.52	6.59	5.00	1.35	1.30	5.44	5.76	23.14
73199	0-8	F	9.06	4.47	7.69	3.75	1.01	1.29	5.38	5.88	23.52
73178	2-0	M	8.40	4.35	7.17	3.75	1.01	1.25	5.23	5.89	23.54
73468	1-0	M	9.03	4.98	7.68	3.75	1.01	1.43	5.99	5.83	23.42
73509	1-4	M	9.70	4.95	7.69	4.00	1.08	1.43	5.99	5.80	23.34
73727	1-4	M	10.33	4.86	6.80	4.75	1.28	1.40	5.85	5.74	23.13
64567	1-6	M	10.99	4.73	5.42	5.75	1.55	1.08	4.52	5.76	23.11
73957	2-6	F	10.33	4.73	7.25	4.50	1.21	1.36	5.69	5.77	23.23
15	0-8	M	11.14	6.25	5.94	5.50	1.48	1.80	7.52	5.57	22.72
16	0-1	F	10.38	6.58	6.75	4.75	1.28	1.90	7.92	5.61	22.87

TABLE D.

Normal children.

79	3-0	M	11.94	5.00	6.21	5.50	1.48	1.44	6.02	5.67	22.92
82	2-6	M	13.17	5.00	7.20	5.75	1.55	1.44	6.02	5.65	22.86
85	1-2	F	13.39	4.47	6.17	6.25	1.69	1.29	5.38	5.66	20.85
91	1-2	M	13.13	4.67	6.87	6.00	1.62	1.35	5.62	5.66	22.87
1ch	12.93	6.48	7.42	5.75	1.55	1.87	7.80	5.53	22.63
2ch	13.58	6.26	7.20	6.00	1.62	1.80	7.53	5.53	22.61
3ch	12.91	6.64	7.80	5.75	1.55	1.91	7.99	5.52	22.61
4ch	12.91	6.30	8.10	5.50	1.48	1.82	7.58	5.57	22.72
1	5-0	F	10.85	4.02	6.92	4.75	1.28	1.16	4.84	5.82	23.30
2	2-0	M	11.55	4.00	7.10	5.25	1.42	1.44	6.02	5.68	23.00
3	3-0	M	11.96	5.43	7.87	5.25	1.42	1.56	6.53	5.64	22.90

The solubility product of $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{=}]^2$ at pH 7.4 expressed as its negative logarithm has been reported to be 23.0. The Graph₁ shows that in 76 out of 78 rachitic cases the values lie above 23.0 indicating slight undersaturation. This should not be an

unexpected finding as the idea that normally the blood serum is supersaturated with regard to $\text{Ca}_3(\text{PO}_4)_2$ is an old one, although the previously accepted pK s.p. $\text{Ca}_3(\text{PO}_4)_2$ was 26.0 to 27.0 (Sendroy and Hastings, *loc. cit.*). Logan and Taylor (1937) and Logan and Kane (1939) found that this value changed to 23.0 in inorganic solutions and blood plasma when the amount of the solid equilibrated with the liquid phase was reduced to a minimum. It will be seen from the reference to the Graph that values for non-rachitic blood serum in all cases except one are below 23.0 indicating that after all the serum might be slightly supersaturated with respect to the salt.

Similarly, with respect to the value of pK s.p. CaHPO_4 there appears to be a demarcation at 5.7, 75 out of 78 values being above 5.7 and 10 out of 11 non-rachitic values lower than 5.7. Shear and Kramer (1928) first suggested that the plasma was undersaturated with respect to CaHPO_4 . The pK s.p. CaHPO_4 , according to them, at pH 7.4 in equilibrated solutions was 5.47. Logan and his associates (*loc. cit.*) by using the methods described above found that the values approached 5.6. Whether the demarcation observed at 5.7 in the figure represents the solubility product indicating saturation or not cannot be defined as no attempt has been made in the present investigation to ascertain the pK s.p. CaHPO_4 experimentally.

Logan (1940) points out that the suggestion of Wendt and Clarke (1923) that the formation of CaHPO_4 was the first step in the formation of bone salt and the findings of Shear and Kramer (*loc. cit.*) mentioned above did not receive the attention they deserved. The reason for this was the fact that at no time the presence of CaHPO_4 in bone could be demonstrated either on the strength of chemical analyses or by X-ray examination of the crystal structure of the bone salt. But the formation of bone salt in stages as described by Logan (*loc. cit.*), for fuller account of which the original article may be consulted, does not exclude such a possibility as that visualized by Wendt and Clarke (*loc. cit.*).

The main object of this paper is to draw attention to the fact that a difference in the state of saturation of the plasma with respect to both the salts, viz. CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$, does exist. Freeman and McLean (1941) have reported their findings on rickets induced in dogs. They state that the serum of rachitic dogs was undersaturated with respect to CaHPO_4 but not necessarily so with $\text{Ca}_3(\text{PO}_4)_2$. That the result of experiments in this laboratory showed undersaturation of serum in rachitic dogs with respect to both of these salts has been reported by Patwardhan and Sukhatankar (1943). Further details of these experiments are being prepared for publication, wherein will also be discussed the significance of these findings and the possibility of using the method of attack as a diagnostic measure in early rickets.

SUMMARY.

1. The blood serum of 79 cases of suspected or frank rickets in infants and children were analysed for calcium, inorganic phosphorus and total protein. Samples of blood serum from 11 normal children were also similarly analysed.

2. The concentration of Ca^{++} , $\text{HPO}_4^{=}$ and $\text{PO}_4^{=}$ at pH 7.4 and the solubility products of $[\text{Ca}^{++}] \times [\text{HPO}_4^{=}]$ and $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{=}]^2$ were calculated from the data.

3. It was found that the pK s.p. values of CaHPO_4 in rachitic cases were over 5.7 in 77 out of 79 cases, whereas in only one out of 11 non-rachitic children was the value above 5.7. With regard to the pK s.p. of $\text{Ca}_3(\text{PO}_4)_2$ 77 out of 79 cases had values above 23.0 showing undersaturation whereas in two cases out of the 11 non-rachitic children the blood was undersaturated with respect to this latter salt.

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NICOTINIC ACID CONTENT OF INDIAN FOODSTUFFS.

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THE pellagra-preventive vitamin was identified with nicotinic acid in 1937 by Elvehjem and his co-workers (Elvehjem, Madden, Strong and Wooley, 1937). Since then nicotinic acid is being widely used in the treatment of pellagra and certain other deficiency states and its importance as a dietary essential has been fully recognized. Hence the determination of the nicotinic acid content of common Indian foods is a subject of great importance. Such knowledge is essential for assessing the nicotinic acid content of diets ordinarily consumed by human beings and also for suggesting diets rich in this vitamin. The investigations so far carried out in India cover only about 50 foods, including some cereals, pulses, fish and animal tissues (Aykroyd and Swaminathan, 1940; Swaminathan, 1938, 1942; Giri and Naganna, 1941; Khorana, Sarma and Giri, 1942; Saha, 1941). No data are available for the nicotinic acid content of many vegetables, fruits and nuts. The present work was undertaken to fill this gap. The nicotinic acid content of about 160 common Indian foods is recorded in this paper. The values already published by the author for about 35 foods are also included (Swaminathan, *loc. cit.*).

METHOD USED.

A chemical method based on König's reaction, for the determination of nicotinic acid in foods has been described by the author (Swaminathan, 1938). Since then numerous modifications of this method have been published by different workers (Shaw and Macdonald, 1938; Bandier, 1939; Harris and Raymond, 1939; Kodicek, 1940; Melnick and Field, 1940*a*, 1940*b*; Dann and Handler, 1941; Giri and Naganna, *loc. cit.*). Further, biological and microbiological methods of assay have also been developed by Waisman, Mickelson, McKibbin and Elvehjem (1940) and by Snell and Wright (1941). Recently, Elvehjem and his co-workers (Waisman *et al.*, *loc. cit.*; Teply, Strong and Elvehjem, 1942) have made a comparative study of the chemical, biological and the microbiological methods and found good agreement between the results obtained. The consensus of opinion is that if due precautions are taken, the chemical method yields reliable results and has further the advantage of being simpler and less time consuming. The method previously published by the author (Swaminathan, 1942), with slight modification, was used in the present study for the estimation of nicotinic acid in foods of vegetable origin. The modification consisted in the decolorization of the extract before hydrolysis, as such a procedure yielded almost colourless or only lightly coloured solutions with all types of food materials. The results obtained with this modification correspond well with those obtained before with the original method and the recovery of nicotinic acid added is good, ranging from 90 to 100 per cent. For animal tissues, fish and yeast, the original method was used.

EXPERIMENTAL.

The method consists of the following steps:—

- (1) Extraction of nicotinic acid from food materials.
- (2) Removal of colouring matter, protein and carbohydrate derivatives, using lead acetate at pH 9 and the excess of lead as lead sulphate.
- (3) Hydrolysis of nicotinamide to nicotinic acid using 2N hydrochloric acid.
- (4) Clarification of the extract with zinc hydroxide at pH 10.
- (5) Colorimetric estimation of nicotinic acid in neutral aqueous medium (at pH 7) using CNBr and aniline.

Extraction.—Five to 100 g. of the finely powdered or minced food material (containing from 0.2 mg. to 1 mg. nicotinic acid) were suspended in 200 ml. of water and the mixture heated in a water-bath for 10 minutes. One ml. of concentrated hydrochloric acid was then added and the heating continued for another 10 minutes with constant stirring. The mixture was then allowed to cool and the volume made up to 400 ml. and centrifuged.

Removal of colouring matter.—Two hundred ml. of the clear extract were taken in a 500 ml. beaker, and 20 ml. of 40 per cent lead acetate solution were then added. The mixture was adjusted to pH 9.4 by the addition of 40 per cent sodium hydroxide (thymol blue as external indicator), and the volume made up to 250 ml. and centrifuged. One ml. of concentrated sulphuric acid was then added to the clear centrifugate to precipitate the excess of lead and the precipitate was removed on the centrifuge.

Hydrolysis of nicotinamide.—One hundred and fifty ml. of the solution from above (corresponding to 3/10 of the original material) were placed in a one-litre beaker. Ten ml. of concentrated hydrochloric acid were then added and the mixture heated in a boiling water-bath for one hour. This resulted in the concentration of the solution to about 80 ml.

Removal of colouring matter with zinc hydroxide.—The solution after hydrolysis was cooled and 5 ml. of 20 per cent zinc sulphate were added. The mixture was brought to pH 10 (phenolphthalein as internal indicator) by the addition of 50 per cent sodium hydroxide. Excess of alkali should be avoided. The volume was then made up to 100 ml. and filtered. The filtrate was adjusted to pH 7 (bromothymol blue as external indicator) by the careful addition of a few drops of 5N and N hydrochloric acid. The solutions were almost colourless in most of the cases. With a few foods, out of 160 so far examined, the final solution was coloured yellow, which was allowed for by a 'blank' determination. One hundred ml. of the final solution correspond to three-tenths of the original material taken.

Colorimetric estimation of nicotinic acid.—Aliquots of the extract (usually 10 ml. = 3/100 of the weight of the material taken) were measured into a series of 25 ml. measuring flasks. Standard nicotinic acid (20 micrograms) was taken in another flask and the volume of the standard diluted to 10 ml. with distilled water. One ml. of 20 per cent sodium acetate solution (adjusted to pH 7) was then added to each flask. Two ml. of 2 per cent aqueous aniline solution were then added to all the flasks, followed by 6 ml. of cyanogen bromide solution. The contents of the flask were mixed and allowed to stand for one minute. The volume was then made up to 25 ml. by the addition of aqueous aniline, the contents of the flasks mixed well and allowed to stand for one minute. The colours were compared (within 10 minutes) in a Klett colorimeter.

A 'blank' estimation was carried out in the above manner for all the coloured extracts, with the difference that distilled water was added instead of cyanogen bromide and aniline. The values so obtained for the 'blank' were allowed for in the usual manner.

By following this procedure, a single worker can determine the nicotinic acid content of about eight foods in about 5 to 6 hours. The results are given in the Table :—

TABLE.
Nicotinic acid content of foods.

Common English name.	Botanical name.	Nicotinic acid (milligrams per 100 g.).
CEREALS AND CEREAL PRODUCTS.—		
Barley, whole	<i>Hordeum vulgare</i>	4.5
Barley, pearl	"	2.8
Italian millet	<i>Setaria italica</i>	0.7
Maize, yellow, dry ..	<i>Zea mays</i>	1.4
Maize, white, dry ..	"	1.3

TABLE—contd.

Common English name.	Botanical name.	Nicotinic acid (milligrams per 100 g.).
CEREALS AND CEREAL PRODUCTS.—contd.		
Maize, white, tender	<i>Zea mays</i>	0·6
Millet (pearl millet)	<i>Pennisetum typhoides</i>	3·2
Millet (kaffir corn)	<i>Sorghum vulgare</i>	1·8
Millet (coracan)	<i>Eleusine coracana</i>	1·4
Oatmeal	<i>Avena sativa</i>	1·1
Rice, raw, husked (prepared in a wooden huller)	<i>Oryza sativa</i>	4·6
Rice, raw, hand-pounded	2·4
Rice, raw, milled	1·2
Rice, parboiled, husked	4·6
Rice, parboiled, hand-pounded	4·0
Rice, parboiled, milled	3·8
Rice, flakes	4·0
Rice, puffed (prepared from paddy)	4·1
Rice, puffed (prepared from rice)	1·1
Rice, polishings, raw	28·4
Wheat, whole	<i>Triticum vulgare</i>	5·0
White flour (refined wheat flour)	1·0
Wholemeal bread	1·8
White bread	0·6
Wheat germ	7·0
PULSES, DRY.—		
Bengal gram	<i>Cicer arietinum</i>	2·6
Black gram	<i>Phaseolus mungo</i>	2·0
Cow gram	<i>Vigna catianga</i>	1·3
Field bean	<i>Dolichos lablab</i>	1·8
Green gram	<i>Phaseolus radiatus</i>	1·7
Horse gram	<i>Dolichos biflorus</i>	1·5
Lentil (massur dahl)	<i>Lens esculenta</i>	1·5
Peas	<i>Pisum sativum</i>	1·3
Red gram	<i>Cajanus indicus</i>	2·4
Soya bean	<i>Glycine hispida</i>	2·4
NUTS AND OILSEEDS.—		
Almond	<i>Prunus amygdalis</i>	2·5
Cashew nut	<i>Anacardium occidentale</i>	2·1
Coco-nut, fresh	<i>Cocos nucifera</i>	0·8
Gingelly seeds	<i>Sesamum indicum</i>	4·4
Ground-nut (pea-nut)	<i>Arachis hypogea</i>	14·1
Mustard	<i>Brassica juncea</i>	4·0
Pistachio	<i>Pistacia vera</i>	1·4
Walnut	<i>Juglans regia</i>	1·6
VEGETABLES, GREEN LEAFY.—		
Amaranth	<i>Amaranthus gangeticus</i>	0·9
Bottle-gourd leaves	<i>Lagenaria vulgaris</i>	0·6
Brussels sprouts	<i>Brassica oleracea bullata gemmifera</i>	0·4
Cabbage	<i>Brassica oleracea capitata</i>	0·4
Carrot leaves	<i>Daucus carota</i>	0·4
Coriander	<i>Coriandrum sativum</i>	0·8
Curry leaves	<i>Murraya koenigii</i>	2·3
Drumstick leaves	<i>Moringa oleifera</i>	0·8
Fenugreek leaves	<i>Trigonella foenum graecum</i>	0·8
Indian spinach	<i>Basella cordifolia</i>	0·5
Ipomea	<i>Ipomoea reptans</i>	0·6
Lettuce	<i>Lactuca sativa</i>	0·4
Mint	<i>Mentha viridis</i>	0·4
Neem, tender	<i>Azadirachta indica</i>	1·4
Parsley	<i>Petroselinum sativum</i>	0·5
Pea leaves	<i>Pisum sativum</i>	0·6
Pumpkin leaves	<i>Cucurbita maxima</i>	0·9
Radish leaves	<i>Raphanus sativus</i>	0·2
Spinach	<i>Spinacia oleracea</i>	0·5

TABLE—contd.

Common English name.	Botanical name.	Nicotinic acid (milligrams per 100 g.).
ROOTS AND TUBERS.—		
Beet root	<i>Beta vulgaris</i>	0·4
Carrot	<i>Daucus carota</i>	0·4
Colocasia	<i>Colocasia antiquorum</i>	0·4
Onion, big	<i>Allium cepa</i>	0·4
Onion, small	"	0·5
Parsnip	<i>Pastinaca sativa</i>	0·4
Potato	<i>Solanum tuberosum</i>	1·2
Radish, white	<i>Raphanus sativus</i>	0·5
Radish, pink	"	0·4
Sweet potato, pink	<i>Ipomoea batatas</i>	0·8
Sweet potato, white	"	0·7
Tapioca, dry	<i>Manihot utilisima</i>	1·2
OTHER VEGETABLES.—		
Ash gourd	<i>Benincasa cerifera</i>	0·4
Bitter gourd	<i>Momordica charantia</i>	0·5
Brinjal	<i>Solanum melongena</i>	0·8
Broad beans	<i>Dolichos lablab</i> var. <i>lignosus</i>	0·8
Cauliflower	<i>Brassica oleracea botrytis</i>	0·9
Cow pea, green and tender (with pod-)	<i>Vigna catiang</i>	0·4
Cucumber	<i>Cucumis sativus</i>	0·2
Drumstick	<i>Moringa oleifera</i>	0·2
French beans	<i>Phaseolus vulgaris</i>	0·3
Indian gooseberry	<i>Phyllanthus emblica</i>	0·2
Jack, tender	<i>Artocarpus integrifolia</i>	0·2
Knol-khol	<i>Brassica oleracea caulorapa</i>	0·5
Lady's finger	<i>Hibiscus esculentus</i>	0·6
Mango, green	<i>Mangifera indica</i>	0·2
Olive, green	<i>Olea europæa</i>	0·3
Papaya, raw	<i>Carica papaya</i>	0·4
Potala gourd	<i>Trichosanthes dioica</i>	0·6
Peas, green	<i>Pisum sativum</i>	0·8
Plantain flower	<i>Musa paradisiaca</i>	0·6
Plantain, green	"	0·3
Plantain stem	"	0·2
Pumpkin yellow, ripe	<i>Cucurbita maxima</i>	0·5
Snake gourd	<i>Trichosanthes anguina</i>	0·3
Sword beans	<i>Canavalia ensiformis</i>	0·5
Tomato green	<i>Lycopersicum esculentum</i>	0·4
Turnip, white	<i>Brassica rapa</i>	0·5
Water chestnut	<i>Trapa bispinosa</i>	0·6
FRUITS.—		
Apple	<i>Pyrus malus</i>	0·2
Banana	<i>Musa sapientum</i>	0·3
Dates, preserved	<i>Phoenix dactylifera</i>	0·8
Grapes	<i>Vitis vinifera</i>	0·3
Grape fruit	<i>Citrus grandis</i> var. <i>maxima</i>	0·3
Guava	<i>Psidium guajava</i>	0·2
Guava, hill	<i>Psidium catehianum</i>	0·3
Hog plum	<i>Spondias mangifera</i>	0·3
Jack fruit	<i>Artocarpus integrifolia</i>	0·4
Lemon juice	<i>Citrus medica</i> var. <i>limonum</i>	0·1
Lime juice	<i>Citrus medica</i> var. <i>acid</i>	0·1
Mango, ripe	<i>Mangifera indica</i>	0·4
Orange juice	<i>Citrus aurantium</i>	0·3
Papaya	<i>Carica papaya</i>	0·2
Peaches, white	<i>Amygdalis persica</i>	0·2
Pears, country	<i>Pyrus communis</i>	0·2
Pears, English	<i>Pyrus achras</i>	0·2
Plantain, ripe	<i>Musa paradisiaca</i>	0·3
Plums	<i>Prunus domestica</i>	0·3

TABLE—concl'd.

Common English name.	Botanical name.	Nicotinic acid (milligrams per 100 g.).
FRUITS.—cont'd.		
Pomelo	<i>Citrus decumana</i>	0.2
Raisins, preserved	<i>Vitis vinifera</i>	0.5
Strawberry	<i>Fragaria grandiflora</i>	0.2
Tomato, ripe	<i>Lycopersicum esculentum</i>	0.4
Tamarind, raw fresh	<i>Tamarindus indicus</i>	0.1
Tamarind, preserved	0.7
Water melon	<i>Citrullus vulgaris</i>	0.2
MILK AND EGGS.—		
Milk, cow's	0.1
Milk, buffalo	0.1
Milk, evaporated	0.2
Milk, sweetened condensed	0.2
Whole milk powder	1.0
Skimmed milk powder	1.1
Eggs, whole, hen's	0.1
Eggs, whole, duck's	0.1
FLESH FOOD.—		
Beef (muscle)	5.9
Fish, specimen 1	3.1
Fish, specimen 2	3.9
Fish, specimen 3 (<i>Calla calla</i>)	1.3
Fish, specimen 4 (<i>Labeo rohita</i>)	2.7
Fish, specimen 5 (<i>Notopterus chitala</i>)	1.0
Liver, sheep	15.3
Liver, rabbit	13.8
Mutton, muscle	6.8
Muscle, rabbit	7.7
Pork, muscle	2.8
CONDIMENTS.—		
Chillies, green	<i>Capsicum annuum</i>	0.5
Coriander seeds	<i>Coriandrum sativum</i>	1.1
Cumin	<i>Cuminum cyminum</i>	2.6
Fenugreek seeds	<i>Trigonella foenum graecum</i>	1.1
Garlic	<i>Allium sativum</i>	0.4
Ginger	<i>Zingiber officinale</i>	0.6
Mustard	<i>Brassica juncea</i>	4.0
Pepper, dry	<i>Piper nigrum</i>	1.4
Tamarind, pulp, preserved	<i>Tamarindus indicus</i>	0.7
Turmeric, dry	<i>Circuma longa</i>	2.3
MISCELLANEOUS.—		
Betel leaf	0.7
Sago	0.2
Jaggery (palmyra)	1.8
Jaggery (sugar cane)	1.0
Yeast, dried, baker's	45.0
Yeast, dried, brewer's 1	62.5
Yeast, dried, brewer's 2	57.1
Yeast, dried, brewer's 3	46.4
Yeast, dried, brewer's 4	43.1
Yeast, dried (grown on molasses salts medium) sample 1.	<i>Torula utilis</i>	26.1
Yeast, dried (grown on molasses salts medium) sample 2.	20.0
Yeast, dried (grown on molasses salts medium) sample 3.	33.7
Yeast, dried, distillery sample 1	9.0
Yeast, dried, distillery sample 2	6.5
'Marmite', an yeast extract	66.5

RESULTS.

Cereals and cereal products.—Among the cereals whole wheat, unmilled rice and whole barley are the richest sources containing about 4 mg. to 5 mg. of nicotinic acid per 100 g. Parboiled milled rice and pearl millet come next in order of merit, with values of 3.8 mg. and 3.2 mg. respectively. The millets, kaffir corn and coracan, oat meal, maize, highly milled raw rice and white flour are poor sources, containing from 1 mg. to 2 mg. Italian millet was found to be the poorest (0.7 mg. per 100 mg.) among the cereals tested. Rice polishings obtained by milling raw paddy is one of the richest known sources, and wheat germ is a good source.

The daily allowances of nicotinic acid for an adult male recommended by the Committee on Food and Nutrition of the National Research Council, U.S.A., range from 18 mg. to 23 mg.

Sixteen ounces of whole wheat, unpolished rice or whole barley will supply about 20 mg. to 25 mg.—almost the daily requirements of the adult—whereas a similar quantity of maize, oats, raw milled rice or white flour will provide only about one-third the amount, i.e. 6 mg. to 7 mg.

Pulses.—Pulses are fair sources, the values obtained for different pulses, ranging from 1.3 mg. to 2.6 mg. per 100 g. A daily intake of four ounces of pulses will contribute only 1.5 mg. to 3.0 mg. of nicotinic acid to the diet.

Nuts and oilseeds.—Of the eight nuts and oilseeds tested, ground-nut (pea-nut) was found to be the richest, containing about 14 mg. per 100 g. Gingelly and mustard seeds rank next in order of merit, with values of 4.4 mg. and 4.0 mg. Other nuts gave lower values. Three ounces of ground-nuts will provide a little more than half the daily adult requirements.

Vegetables.—Vegetables were found to be poor sources of nicotinic acid. In general leafy vegetables, roots and tubers were found to be richer than other vegetables. A daily intake of eight ounces of vegetables (four ounces leafy and four ounces non-leafy) will provide about 1 mg. to 2 mg. of nicotinic acid.

Fruits.—Fruits are poor sources, containing from 0.1 mg. to 0.5 mg. per 100 g. A daily intake of four ounces of fruits will supply only 0.2 mg. to 0.5 mg. of nicotinic acid.

Condiments.—Condiments are in general fair sources. Since they are included in human diets only in small quantities, the contribution of nicotinic acid by this group of foods is negligible.

Milk and eggs.—Contrary to the general belief, milk and eggs are very poor sources of nicotinic acid. Similar values have been reported by Elvehjem and his co-workers using different methods of assay (Waisman *et al.*, *loc. cit.*; Teply *et al.*, *loc. cit.*). A daily intake of 1 pint of milk or 2 eggs will provide only 0.6 mg. and 0.1 mg. (i.e. negligible quantities) of nicotinic acid respectively.

Liver.—Mammalian liver is one of the richest known sources containing from 12 mg. to 18 mg. per 100 g. Four ounces of liver will supply almost the daily adult requirements of this vitamin.

Flesh foods.—Among the flesh foods, meats are good sources; but their nicotinic acid content varies, depending on the source. Thus, mutton and beef contain about twice as much as pork. Fish is only a fair source, the values obtained for different fishes varying from 1 mg. to 4 mg. Eight ounces of mutton will supply almost the daily requirements for an adult.

Dried yeast.—Dried yeast, though not a common article of food, is a valuable supplement to the human dietary, as a source of the vitamin B complex. Hence it was felt to be of interest to assay yeast prepared by different methods for their nicotinic acid content. The results show that dried brewer's yeast is a better source of nicotinic acid than food yeast (*Torula utilis*), grown on molasses salts medium. The values obtained ranged from 43 mg.

to 62 mg. and 20 mg. to 34 mg. per 100 g. of dried brewer's yeast and food yeast respectively. Distillery yeast, a by-product in the manufacture of alcohol by the fermentation process, is a poor source containing 7 mg. to 9 mg. per 100 g.

The therapeutic doses of yeast and nicotinic acid in the treatment of pellagra.—Goldberger and Lillie (1926) used a daily dose of 30 g. of dried yeast in the treatment and prevention of pellagra; but more recently Spies *et al.* (1937) found it necessary to use larger quantities of dried brewer's yeast (180 g. to 270 g.) in addition to a well-balanced diet of 4,500 calories containing large amounts of milk, eggs and meat, in the treatment of severe endemic pellagra. Such a diet, supplemented by yeast, would supply about 200 mg. of nicotinic acid.

Recent investigations of Spies and his co-workers (1939) have shown that 500 mg. to 1,000 mg. of nicotinic acid are required daily by the oral route for the effective treatment of severe endemic pellagra, while as little as 50 mg. daily may be effective when administered parenterally. Smaller doses may be effective in some mild cases.

The introduction of nicotinic acid has been a real advance in the treatment of pellagra. Spectacular improvement is now seen within 24 hours and cases are usually free of signs and symptoms within a week. With the old dietetic treatment alone, progress was always slow and it was often many weeks before recovery was complete. Further, a proportion of cases did not respond to the improved diet and died. It is probable that the richest diets used up to 1937 could supply no more than one quarter (200 mg.) of the amount of nicotinic acid now found necessary for the maximum rate of recovery.

SUMMARY.

1. The nicotinic acid content of about 160 foodstuffs has been determined by an improved chemical method using the cyanogen bromide and aniline reagent.
2. Of the foods tested, dried brewer's yeast, liver, rice polishings (from raw paddy) and pea-nuts are rich sources.
3. Meats of various kinds are good sources and fish a fair source of nicotinic acid.
4. Among the cereals, whole wheat, unmilled rice, whole barley, parboiled rice and pearl millet are good sources, containing 2 to 3 times as much nicotinic acid as white flour, maize, oatmeal, certain millets and raw milled rice. Maize, which is known to be associated with pellagra, gave a low value; but similar low values were also obtained for white flour, oats, milled raw rice and certain millets. Italian millet contained only half as much nicotinic acid as that found in whole maize.
5. Cow's milk, eggs, fresh vegetables and fruits are in general poor sources of nicotinic acid.

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THE INCIDENCE OF PERIODONTAL DISEASE IN THE PUNJAB.

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CASUAL observations over a number of years in India would seem to indicate a very high incidence of periodontal diseases. The whole range of paradental disease is represented, from the very prevalent gingivitis in children to the almost universal chronic suppurative periodontitis of adult life. Recent more specific surveys have established the great prevalence of the hypertrophic type of gingivitis in young children and adolescents and of a condition of chronic alveolar bone-absorption in adolescents and young adults with or without the presence of suppuration and pocket-formation. Amongst the middle-aged and in later life an Indian mouth showing a complete absence of active pus-formation is rarely found.

Though many theories have been advanced from time to time the ætiology of periodontal conditions has never been satisfactorily explained. Whether these conditions are due to specific dietary deficiencies, faulty body-metabolism, lack of proper oral hygiene, traumatic occlusion or other factors has never been adequately established. In many cases it would seem that some of these periodontal conditions occur as manifestations of various systemic diseases. It is highly probable as in the case of dental caries that there is not a single specific ætiological factor but rather several factors operating together to produce this breakdown in the oral tissues.

A recent survey in a local hospital showed that 90 of the 100 patients examined in the medical and surgical wards had periodontal disorders with active pus-formation. A very high percentage of the 38,000 patients attending annually in the Punjab Dental Hospital, Lahore, is similarly affected. Whatever the cause, it is possible that much of the ill health in the province has its origin in neglected oral infection and that many hospital beds are kept occupied perhaps for long periods because oral infection is often entirely disregarded as a possible cause or at least a contributory cause of many systemic conditions. It seems obvious that the presence of a large area of suppurating tissue at the opening of the alimentary tract in a high percentage of the population must constitute a very serious threat to the health of the community.

As a preliminary to a wider investigation of periodontal disease in India it seemed desirable in the first instance to obtain some reliable data relating to the incidence of the disease. In the present paper some observations of the prevalence of the disease in the Punjab are recorded.

METHOD OF INVESTIGATION.

A complete examination of gingival and periodontal tissues was made with mirror and probe. In the diagnosis of gingivitis and pyorrhœa, hypertrophy of the gums, the depth of the gingival pockets, suppuration and discharge of pus from the pockets and the mobility of the teeth were noted. In the early stages of gingivitis there is slight thickening of the gums, while in the later stages there is marked hypertrophy with marginal ulceration and pocket-formation. Pyorrhœa is a chronic inflammatory process which is commonly associated with absorption of alveolar processes, pocket-formation and loosening of the teeth. Suppuration

and discharge are usually present. There are, however, certain conditions to which the term pyorrhœa has also been applied, e.g. senile atrophy of the gums and pathological absorption, in which there is no suppuration or discharge.

In the present investigation cases were grouped under the heads 'gingivitis' and 'pyorrhœa'. It is to be observed that severe cases of gingivitis are difficult to distinguish from pyorrhœa, so that there was no sharp line of demarcation between the two groups. Most probably pyorrhœa is a later manifestation of a continuous pathological process of which gingivitis is an early sign.

Cases of pyorrhœa were further classified into two groups with the following characteristics:—

(a) General horizontal absorption of the alveolar processes with pocket-formation and discharge of pus. This corresponds to the 'dirt pyorrhœa' of Fish (1935).

(b) Localized absorption of the alveolar processes in the neighbourhood of a few individual teeth, or groups of teeth, with pocket- and pus-formation. This condition corresponds to Fish's 'idiopathic pyorrhœa'.

Radiological examination is of great value in the study of periodontal disease, but owing to the shortage of X-ray films it could not generally be employed in the present investigation. A complete radiographic examination was, however, made of 65 cases.

GROUPS STUDIED.

A. 996 hospital patients. These were in-patients in the Mayo Hospital, Lahore, and out-patients attending the Conservation Department of the Punjab Dental Hospital, Lahore.

B. 1,074 healthy male adults (Punjabi police constables) of active habits.

C. 613 school children attending two schools in Lahore.

RESULTS.

Tables I and II show that the observed incidence of gingivitis and pyorrhœa was very high. The percentage of clinically normal persons was 2·8, 20·5 and 12·1 in the hospital patients, the school children and the police constables respectively. Agnew and Agnew (1941) reported a similar high incidence of periodontal disease and hypertrophic gingivitis in China, though they did not carry out systematic surveys.

TABLE I.

The incidence of gingivitis and pyorrhœa in patients in the Punjab Dental Hospital and the Mayo Hospital, Lahore.

Age-group.	Number examined.	Clinically normal, per cent.	Gingivitis, per cent.	Pyorrhœa, per cent.
5 to 10	.. 16	12·5	81·2	6·2
11 to 20	.. 220	5·4	56·8	37·2
21 to 30	.. 383	2·8	30·0	67·1
31 to 40	.. 202	1·0	12·3	86·6
41 to 50	.. 122	..	4·1	95·8
51 to 60	.. 45	..	2·2	97·8
60 and above	.. 18	100·0
TOTALS	.. 996	2·8	28·5	68·6

Percentage of 'dirt' pyorrhœa = 81·2.

Percentage of 'idiopathic' pyorrhœa = 18·8.

TABLE II.

A.

Incidence of periodontal disease in 613 school children.

Age-group.		Number examined.	Normal, per cent.	Gingivitis, per cent.	Pyorrhœa, per cent.
5 to 10	..	196	19·8	74·4	5·3
11 to 15	..	417	20·8	73·2	5·7
TOTALS	..	613	20·5	73·7	5·7

B.

Incidence of periodontal disease in 1,074 police constables in the Punjab.

Age-group.		Number examined.	Normal, per cent.	Gingivitis, per cent.	Pyorrhœa, per cent.
18 to 20	..	225	21·3	42·2	36·4
21 to 30	..	525	10·2	27·2	62·4
31 to 40	..	215	9·7	9·7	80·4
41 to 50	..	101	6·9	1·9	91·0
51 to 60	..	8	100·0
TOTALS	..	1,074	12·1	24·3	63·6

Percentage of 'dirt' pyorrhœa = 82·5.

Percentage of 'idiopathic' pyorrhœa = 17·5.

The incidence of gingivitis decreased with age while that of pyorrhœa increased. In the older age-groups the percentage suffering from pyorrhœa was over 90. The frequency with which pyorrhœa was observed in children is remarkable and contrary to experience in Western countries. The age incidence of the two conditions definitely suggests that hypertrophic gingivitis among children progresses into pyorrhœa later in life.

So-called 'dirt' pyorrhœa as defined above was much more common than 'idiopathic' pyorrhœa. The relative incidence of both kinds of pyorrhœa was fairly constant throughout the different age-groups.

Sex incidence of periodontal disease.—Table III shows the incidence in male and female hospital patients. Except in the age-group 21–30 years there was no statistically significant difference in the percentage incidence of gingivitis and pyorrhœa in the two sexes. The significant difference, to the advantage of women, in the age-group in question may be due to the fact that women pay more attention to the care of their teeth and gums during this period of life, and is an observation of some interest.

TABLE III.

Sex incidence of gingivitis and pyorrhœa (hospital patients).

Age-group.		Number examined.	Clinically normal, per cent.	Gingivitis, per cent.	Pyorrhœa, per cent.	Whether signifi- cant or not.
5-10	.. {	M 10	10.0	80.0	10.0	} Not significant.
		F 6	16.6	83.3	3.0	
11-20	.. {	M 140	2.8	57.8	39.2	} " "
		F 80	11.2	55.0	33.7	
21-30	.. {	M 291	3.4	24.7	71.7	} Significant.
		F 92	1.0	46.7	52.7	
31-40	.. {	M 148	0.6	11.5	87.8	} Not significant.
		F 51	1.8	11.8	83.3	
41-50	.. {	M 101	..	3.8	96.1	} " "
		F 18	..	5.5	94.3	
51-60	.. {	M 38	100.0	}
		F 7	..	14.2	85.8	
61 and above	.. {	M 8	100.0	}
		F	
TOTALS	.. {	M 739	2.1	24.7	73.1	
		F 257	4.6	39.3	56.1	

Incidence in vegetarians and non-vegetarians.—There is a general impression in India that meat-eating leads to pyorrhœa. Examination of the incidence of periodontal disease in vegetarians and non-vegetarians does not support this view (Table IV). The differences in all age-groups were not significant. Further, the incidence of pyorrhœa is higher in Indians than in Europeans and Americans whose intake of meat is much larger.

The significance of the differences in the various groups was determined by calculating with the help of a fourfold table, $P = 0.05$, being taken as the conventional level of significance (Hill, 1937).

TABLE IV.

Incidence of periodontal disease in vegetarians and non-vegetarians.

Age-group.		Number examined.	Normal.	Gingivitis.	Pyorrhœa.	Whether signifi- cant or not.
5-10	.. {	Vegetarians 4	1	3	..	} .
		Non-vegetarians 10	2	7	1	
11-20	.. {	Vegetarians 52	5	27	20	} Not significant.
		Non-vegetarians 130	6	76	48	
21-30	.. {	Vegetarians 57	..	21	36	} " "
		Non-vegetarians 274	9	68	197	
31-40	.. {	Vegetarians 20	..	3	17	} " "
		Non-vegetarians 167	1	19	147	
41-50	.. {	Vegetarians 12	12	} " "
		Non-vegetarians 106	..	5	101	
51-60	.. {	Vegetarians 14	..	1	13	} " "
		Non-vegetarians 30	30	
61 and above	.. {	Vegetarians	
		Non-vegetarians 7	7	
All ages	.. {	Vegetarians 159	Per cent. 3.7	Per cent. 34.6	Per cent. 61.6	
		Non-vegetarians 724	2.4	25.5	73.6	

Radiographic examination.—Thirty-six Indians and 29 Europeans and Anglo-Indians were examined. In the Indian group the incidence of pyorrhœa as estimated by radiography was 100 per cent (55·6 per cent, 'dirt' pyorrhœa; 44·4 per cent, 'idiopathic' pyorrhœa). Of the 29 patients in the other group, 3 were normal, while 18 (62·0 per cent) and 8 (27·6 per cent) showed 'dirt' pyorrhœa and 'idiopathic' pyorrhœa respectively. Radiographic examination revealed a higher incidence than was evident on clinical examination.

DISCUSSION.

The object of this preliminary investigation was to study the incidence of periodontal disease and little light has as yet been thrown on the problem of ætiology. In a previous paper (Day and Shourie, 1943) the authors reported that the daily administration of 50 mg. of vitamin C for 100 days had no effect on gingivitis in children. The present investigation has, however, produced some useful data about the incidence of gingivitis and pyorrhœa. It is clear that the ætiology of these very common conditions merits further detailed and careful investigation.

SUMMARY.

1. The incidence of gingivitis and pyorrhœa in hospital patients, police constables and school children in Lahore has been investigated. Both conditions were found to be very common. The frequency of gingivitis decreased with age and that of pyorrhœa increased, so that the latter appears to be a later stage of the former.

2. Except in the age-group 21–30, the incidence was similar in both sexes. In the age-group in question, females showed less periodontal disease.

3. The incidence of periodontal disease was similar in vegetarians and non-vegetarians.

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GENERAL PHARMACOLOGY OF *UMBELLATINE*, A NEW ALKALOID
ISOLATED FROM *BERBERIS UMBELLATA* WALL. AND
BERBERIS INSIGNIS HOOK. F. AND ITS USE IN
THE TREATMENT OF ORIENTAL SORE.

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- INTRODUCTION.

MOST of the species of *Berberis* (N. O. Berberidaceæ) contain *Berberine* and some of them contain, in addition, other alkaloids (Chopra, 1933). Chatterjee (1940, 1941) found no *Berberine* in the two Himalayan species—*Berberis umbellata* Wall. and *Berberis insignis* Hook. f. He could detect only one alkaloid in these two species. On analysis this proved to be a new alkaloid which has been named *Umbellatine* by Chatterjee. The chemical properties and structure are being investigated by him. The formula of *Umbellatine* as suggested by him is $C_{21}H_{25}O_3N$ and the accepted formula of *Berberine* is $C_{20}H_{19}O_5N$. Chatterjee (private communication) has adduced evidence to prove the presence of four OH groups and one N.Me group in addition to two O.Me groups which are present in *Berberine*. In place of the N.Me group of *Umbellatine*, *Berberine* contains the NH group.

The general pharmacology of *Berberine* has been investigated by Chopra, Dikshit and Chowhan (1932). Seery and Bieter (1940) have demonstrated the protective action of the alkaloid against *Trypanosoma equiperdum* infection and also the anæsthetic action of the drug. Das Gupta and Dikshit (1929) demonstrated the inhibitory action of the drug on cultures of *Leishmania tropica*, thus proving the rationale of the use of the drug first suggested and worked out by Verma (1927). *Berberis*, known in the indigenous system of medicine as '*Daruharidra*', has been used for a long time as a febrifuge, a carminative and as a healing ointment in indolent ulcers. This last use prompted Jolly (1911) to use a dried extract of *Berberis* (Rasaut) in the treatment of oriental sore. Subsequent workers—Karamchandani (1927) and Lakshmi Devi (1927), to name only two—have assured for *Berberine* a definite place in the chemotherapy of oriental sore. Bruno Putzer (1931), in his attempts to find a more potent derivative of *Berberine* in the treatment of oriental sore, synthesized many compounds and tried them in experimental oriental sore. He came to the conclusion that only soluble products of *Berberine* in the ammonium form have any chemotherapeutic effect.

Apart from the general pharmacological interest, a pharmacological and therapeutic investigation of *Umbellatine* was undertaken to find if the structural modifications present in *Umbellatine* have altered its actions.

Chemistry.—*Umbellatine* is a crystalline base more yellow than *Berberine*. The base is more soluble than any of its salts (chloride, sulphates, nitrates, etc.). At about the body temperature a solution of approximately 0.5 per cent can be made. Like *Berberine*, the base is thrown out of solution in the presence of electrolytes (normal saline and blood, etc.). In solution *Umbellatine* is positively charged and moves to the cathode. With urea a more soluble product can be made.

Experimental.—In the following biological experiments a 0·5 per cent solution has been used. A comparison with *Berberine* (0·5 per cent solution of the base was used) was made simultaneously.

General protoplasmic action.

On paramæcium.—*Umbellatine* 1 in 500 impairs the activity of paramæcium in 3 minutes and kills these protozoa in 30 minutes. The time required for the sub-lethal and lethal action is less than that required by *Berberine* in the same concentration.

Specific action.—*Umbellatine* 1 in 50,000 inhibits the growth of *Leishmania tropica* in liquid hæmoglobin saline medium. In 1 in 100,000 concentration of *Umbellatine*, growth was very scanty, and in a culture examined after 12 days all the flagellates were found dead. *Berberine* inhibits the growth of *Leishmania tropica* in a dilution of 1 in 80,000 (Das Gupta and Dikshit, *loc. cit.*). The growth of *Leishmania donovani* and *Entamoeba histolytica* is not inhibited even in 1 in 10,000 dilution. The effect of various drugs on the growth of *E. histolytica*, *L. tropica* and *L. donovani* is being investigated further.

Local effects.—When dropped in the eye, the conjunctival vessels dilate in rabbits, guinea-pigs and cats, the dilatation being more marked than with *Berberine* of the same strength. The corneal reflex is retarded; with *Berberine* no such retardation was evident. Urea *Umbellatine* (1 per cent solution) causes more congestion and loss of corneal reflex. Injected subcutaneously in experimental animals, *Umbellatine* causes a dilatation of the local vessels (in man, and in the rabbit's ear, the area injected becomes pink). There is no irritation or tissue death; there is slight dulling of sensation in the injected part. The drug is absorbed both from subcutaneous and intramuscular injection, no trace of the drug being visible at the site of inoculation 24 hours after injection.

Circulation.—In cats under anæsthesia with chloralose or urethane, *Umbellatine* in 2 mg./kg. dose given intravenously causes a fall of blood pressure, the range of fall (20 to 30 per cent) depending on the initial level of blood pressure and the dose used. With 2 mg./kg. dose, return to the original level occurs within 2 to 3 minutes. *Berberine* in some doses causes a greater fall (30 to 40 per cent) and the return to the original level takes a longer time.

After paralysis of the parasympathetic nerve-endings with atropine, the fall of blood pressure due to *Umbellatine* as well as that due to *Berberine* is less.

In spinal cats also, *Umbellatine* causes a similar fall of blood pressure. The cat's heart *in situ* reacts by a diminution of contractility which is shared by both ventricle and auricle. This is similar to the action of *Berberine*.

The abdominal organs, e.g. spleen, intestine and kidney, dilate during the fall of blood pressure, i.e. there is simultaneous splanchnic dilatation as the blood pressure falls. As the blood pressure gradually rises to normal, the spleen, intestine and kidney vessels constrict and their volumes diminish. *Berberine* also acts similarly.

Smooth muscles.—With *Umbellatine* in 2 mg./kg. dose the spleen exhibits increased rhythmic movements due to the rhythmic contraction of the splenic capsular muscle.

Intestine.—In cats under chloralose anæsthesia, the intestinal movements, recorded with Jackson's enterograph, show increased tonicity, contractility and rhythmicity persisting for about 15 minutes after 2 mg./kg. dose given intravenously. A similar action was observed with *Berberine* but the duration and intensity were much less with the same dose.

Uterus.—The effect on the uterus was studied *in situ* by Barbour's technique in cats under chloralose anæsthesia. Strips of the uterus of the virgin guinea-pig were suspended in oxygenated Fleisch's solution in a Dale's bath. Under both of these conditions the uterine muscle responded by increased contraction and tone. The effects *in situ* preparations were more marked, the dose being 2 mg./kg. The dilution in the bath was about 1 in 150,000. The effect with *Umbellatine* was on the whole more intense than that with *Berberine*.

Bladder.—The contractions were recorded by a tambour device connected to a cannula introduced into the bladder through its anterior wall and fixed in position by purse-string

suture. *Umbellatine* (2 mg./kg.) produced sustained contraction of the bladder. *Berberine* also produced a similar contraction but it was much less. After paralysis of the parasympathetic nerve-endings with atropine, the stimulating action of *Umbellatine* or *Berberine* is much less or even absent.

Respiration.—In cats under urethane anaesthesia the respiratory movements were recorded with a tambour device attached to the tracheal cannula. With *Umbellatine* (2 mg./kg.), the respiratory rate increases as the blood pressure falls, and it soon diminishes to about half the original rate. Bronchi first dilate and then contract as the rate falls. *Berberine* also depresses the respiration similarly.

DISCUSSION.

The fall of blood pressure shown in spinal cats proves that it is not due to any central effect. The presence of splanchnic dilatation, conjunctival congestion, and dilatation of blood vessels observed at the site of injection, point towards a direct dilating action on the vessels. The diminished fall of blood pressure after paralysis of the parasympathetic nerve-endings by atropine suggests that it may be due partly to the stimulation of the parasympathetic dilators. The nerve-depression of the heart which obviously is due to direct action on the cardiac muscle also contributes to the fall of blood pressure.

The smooth muscles of the uterus, intestine, bladder and spleen are stimulated. The relatively diminished action on the bladder after atropine suggests that in some of these organs at least the increased activity observed is due to parasympathetic stimulation.

Umbellatine resembles *Berberine* in all its actions but it is more active than *Berberine* except in producing cardiovascular responses. This increased effect of *Umbellatine* may be due to the four hydroxy groups it possesses. May and Dyson (1939) remark that 'in the aromatic compounds the entrance of hydroxyl groups usually causes an increase in both the physiological effect and toxicity'. The diminished cardiovascular effect of *Umbellatine* is very probably due to the replacement of hydrogen of NH group of *Berberine* by the methyl radical to form NCH₃ group in *Umbellatine*.

Therapeutic use.—Prompted by the close similarity of chemical structure of *Umbellatine* to *Berberine* and its inhibitory action on the growth of *Leishmania tropica*, we have used *Umbellatine* in the treatment of 6 cases of oriental sore. These patients had multiple sores (more than three), the majority of which were secondarily infected. The sores were locally infiltrated with *Umbellatine* (0.5 per cent solution) following the same technique as suggested for the treatment of oriental sore by *Berberine* acid-sulphate solution. The small uninfected sores were healed with one or two injections. The infected sores required as many as 10 to 12 weekly injections. No advantage accrues by diminishing the interval between injections. In one case two secondarily infected sores of almost equal size were simultaneously treated separately with *Umbellatine* (0.5 per cent) and Orisol (2 per cent *Berberine* acid sulphate); these two sores required the same number of injections of Orisol and *Umbellatine* for healing. The results so far achieved are encouraging. In one case we used urea *Umbellatine* (1 per cent) with very good results. Further work is in progress.

SUMMARY AND CONCLUSION.

1. *Umbellatine*, like *Berberine*, is not a protoplasmic poison.
2. It has a specific inhibitory action on the growth of *Leishmania tropica*—the causative organism of oriental sore.
3. It has a depressant action on the cardiovascular system. The heart is depressed directly. Blood vessels are dilated directly and also perhaps by stimulation of the parasympathetic vasodilator nerve-endings.
4. The smooth muscles of spleen, intestine, uterus and bladder are stimulated. In some of these the action seems to be due to the stimulation of the parasympathetic nerve-endings of the muscles concerned.
5. Respiration is depressed and bronchi are constricted.

6. *Umbellatine* has been used with success in the treatment of oriental sore.

7. The pharmacological actions of *Umbellatine* are similar to those of *Berberine* but more intense except in producing cardiovascular responses which are less than those caused by *Berberine*.

8. Salts of *Umbellatine* are less soluble than the base itself. With urea a more soluble product has been obtained.

Our best thanks are due to Mr. R. Chatterjee, Professor of Chemistry, St. Joseph College, Darjeeling, for a liberal supply of the drug.

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INFLUENCE OF PHENOLS ON PHENOL REAGENT.

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THE authors (Ray and Basu, 1943) have shown that p-chloro-m-cresol reduces phenol reagent and that, from the intensity of the blue colour formed, the amount of chlorocresol present in an unknown solution can be estimated. It was observed that the reducing capacity of chlorocresol was lower than that of pure phenol. To find out whether this is a characteristic of chlorocresol or whether the reducing capacity of a phenol is dependent on the substituents present in the phenol molecule, the behaviour of different phenols with phenol reagent was studied. It has been found that the nature, as well as the position of the substituent in the phenol molecule, plays a considerable part in altering the reducing power of the phenol. If this factor be taken into account, various phenols can be estimated against a standard phenol from Beer's formula :—

$$C_1 = \frac{R_2}{R_1} \times C_2 \times \frac{100}{V} \times \frac{\text{Mol. weight of unknown phenol}}{\text{Mol. weight of pure phenol}}.$$

Certain substituents again completely inhibit the reducing capacity of phenols.

Reaction with phenol reagent.—The phenol reagent was prepared as described by Ray and Basu (*loc. cit.*). Pure phenol was standardized by bromide-bromate solution, and used as standard phenol for colorimetric comparison. A certain volume of the standard phenol solution and a suitable volume of the unknown solution containing almost a molar amount of the phenol to be tested were placed separately in two different 100 c.c. volumetric flasks. To each, 2 c.c. of the phenol reagent and 2 c.c. of a 20 per cent solution of sodium carbonate were added. The volume in each flask was made up to 100 c.c. and the blue colour which developed in each was compared in a Klett colorimeter using Beer's formula, where

C_1 = Concentration per 100 c.c. solution of the phenol tested,

C_2 = Amount of the standard pure phenol,

R_1 = Reading of the solution under test,

R_2 = Reading of the standard phenol,

V = Volume of the solution under test.

P-chloro-m-cresol.—Three different solutions containing 0.10064 g., 0.10110 g. and 0.10690 g. of chlorocresol were made in water and the volume in each case was made up to 100 c.c., and each solution was then treated with the phenol reagent. The blue colour which developed was compared immediately against that developed by standard phenol solution containing 0.553 g., 0.467 g. and 0.500 g. of phenol respectively. The molar ratio between chlorocresol and phenol is 1.516. From the above formula the respective percentages of chlorocresol came to 0.08818, 0.08944 and 0.09475 against 0.10064, 0.10110 and 0.10690. In each case the chlorocresol was found to be lower than the amount taken. Thus chlorocresol reduces the phenol reagent to a lower degree than phenol itself. From Table I, it will be seen that this factor comes to 1.129; in other words, 1.129 mole of chlorocresol produces a colour intensity which is equivalent to that produced by one molecule of pure phenol.

p-cresol.—A 0.7254 per cent solution of para-cresol was made with water. This solution (0.1 c.c.) was placed in a 100 c.c. volumetric flask and 0.1 c.c. of 0.4747 per cent standard phenol solution was placed in another volumetric flask of the same capacity. Both were treated with the phenol reagent as usual and the reducing capacity of *p*-cresol observed. Two similar additional tests were made with different amounts of standard phenol. The results of the experiments are recorded in Table II. From the average of three experiments it is found that the reducing capacity of 1.063 mole of *p*-cresol is equivalent to that of pure phenol.

m-cresol.—Three sets of experiments were similarly made with 0.1 c.c. of a 0.3696 per cent *m*-cresol solution against different amounts of standard phenol and the results recorded in Table III. In this case the amount found was greater than the amount actually taken. The factor comes to 0.965.

p-chlorophenol.—A 0.2468 per cent solution of *p*-chlorophenol was prepared. Three colorimetric estimations were made with 0.30 c.c., 0.40 c.c. and 0.31 c.c. of the above solution against three sets of standard phenol solution respectively. The results are recorded in Table IV. It will be observed that 1.238 molecule of *p*-chlorophenol reduces an amount of phenol reagent equal to that reduced by only one molecule of pure phenol.

p-methoxy phenol, *p*-hydroxy benzoic acid, and ethyl *p*-hydroxy benzoate were also treated with the phenol reagent but the intensity of colour developed was negligible. The influence of a more negative substituent like 'nitro' group was also studied. *m*-nitrophenol developed a colour intensity far below that produced by the normal phenols. With *p*-nitrophenol no colour developed. Hydroquinone, on the other hand, reduced phenol reagent instantaneously even before the addition of any alkaline reagent.

TABLE I.

Experiment.	R ₁ .	R ₂ .	V, c.c.	C ₂ , g.	C ₁ , g.		Ratio, T/F.	Average factor.
					Found, F.	Taken, T.		
1	23.6	20	0.8	0.000553	0.08818	0.10064	1.1285	..
2	19.8	20	0.8	0.000467	0.08944	0.10110	1.1300	1.129
3	20.0	20	0.8	0.000500	0.09475	0.10690	1.1283	..

TABLE II.

1	16.0	20	0.1	0.0004747	0.6824	0.7254	1.063	..
2	15.7	20	0.1	0.0004670	0.6841	0.7254	1.060	1.063
3	22.5	20.	0.1	0.0006646	0.6794	0.7254	1.067	..

TABLE III.

1	18.7	20	0.1	0.0003115	0.3831	0.3696	0.9640	..
2	24.3	20	0.1	0.0004050	0.3832	0.3696	0.9640	0.965
3	20.6	20	0.1	0.0003426	0.3825	0.3696	0.9663	..

TABLE IV.

Experiment.	R ₁ .	R ₂ .	V, c.c.	C ₂ , g.	C ₁ , g.		Ratio, T/F.	Average factor.
					Found, F.	Taken, T.		
1	23.6	20	0.30	0.000514	0.1983	0.2468	1.244	..
2	19.4	20	0.40	0.000561	0.1976	0.2468	1.249	1.238
3	20.8	20	0.31	0.000467	0.2020	0.2468	1.222	..

TABLE V.

*Estimation of different phenols behaving normally with phenol reagent.
Standard solution : pure phenol.*

Substance.	R ₁ .	R ₂ .	V, c.c.	C ₂ , g.	F.	C ₁ , g.		Error, per cent.
						Found.	Taken.	
Chlorocresol ..	19.8	20	0.80	0.0004670	1.129	0.1010	0.1011	-0.10
P-cresol ..	16.0	20	0.10	0.0004747	1.063	0.7254	0.7254	Nil.
M-cresol ..	18.7	20	0.10	0.0003115	0.965	0.3697	0.3696	0.03
P-chlorophenol	20.8	20	0.31	0.0004670	1.238	0.2500	0.2468	0.08

DISCUSSION.

From the above experiments it is evident that the substituent in the phenolic compound plays a considerable part in its reducing action on the molybdate molecule on which depends the development of colour when a phenolic reagent is treated with a phenol. The more negative the substituent the less is the reducing capacity of the phenol. Thus, p-nitrophenol does not develop any colour with phenol reagent. This inhibitory action follows the order: $\text{NO}_2 > \text{COOH} > \text{COOR} > \text{OCH}_3 > \text{Cl} > \text{CH}_3 > \text{OH}$. The last compound, hydroquinone, rapidly reduces the phenol reagent. The position of the substituent again plays a part in this direction: thus, the nitro group, when present at the para position, completely inhibits the reducing capacity of phenol, while with meta-nitrophenol some reduction is observed. The influence of position is also apparent in the case of a methyl substituent. The reducing capacity of 1.063 mole of p-cresol is equivalent to one mole of phenol, but when this methyl group is shifted to the meta position as in m-cresol, the reducing capacity of this substituted phenol increases, as is evident from the results recorded in Table III. Less than one mole of m-cresol is required to reduce that amount of the reagent which is required by one mole of pure phenol. The phenols used in these investigations were those available in the market and were unpurified.

Phenols which behave normally in reducing phenol reagent can easily be estimated by a colorimetric method using Beer's formula. The molar ratio between the phenol to be estimated and standard phenol has to be multiplied by an ascertained factor, i.e. the reducing capacity of the unknown phenol. The formula thus becomes—

$$C_1 = \frac{R_2}{R_1} \times C_2 \times \frac{100}{V} \times \frac{\text{Mol. weight of phenol to be tested}}{\text{Mol. weight of pure phenol (standard)}} \times F$$

(F being the respective reducing capacity of phenol to be tested). From Table V it will be evident that chlorocresol, p-cresol, m-cresol and p-chlorophenol can easily be estimated against a standard phenol and the percentage of error is practically negligible.

CONCLUSION.

The behaviour of phenols with phenol reagent is dependent on the nature and position of the substituent in the phenol molecule. Nitro group, when present in the para position, does not reduce phenol reagent, whereas the hydroxy group in the same position enhances the reducing capacity to a very considerable extent.

P-chloro-m-cresol, p-cresol, m-cresol, and p-chlorophenol can easily be estimated in solution with a phenol reagent.

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STUDIES ON SULPHANILYL-BENZAMIDE: TOXICITY AND ABSORPTION.

BY

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EVEN before the introduction of sulphanilyl-guanidine by Marshall *et al.* (1940, 1941) against intestinal bacillary infections, attempts in this direction were already being made by other workers with sulphanilamide, sulphapyridine and sulphathiazole (Jones and Abse, 1939; Gorlitzer, 1940; Drobinskiy, 1940; Cooper and Keller, 1940; Ching *et al.*, 1940; Haldo and Devincenzi, 1940). Sulphanilamide and its derivatives, being rapidly absorbed, were quickly removed from the intestines, without reaching a prolonged destructive concentration against the pathogenic organisms. The value of sulphanilyl-guanidine lay in its poor absorption from the gut, its solubility in water, and its high antibacterial activity. Brownlee and Tonkin (1941), working on derivatives of the sulphanilamide and sulphone class which possess similar characteristics, reported the efficacy of sulphanilyl-benzamide *in vitro* against organisms of the dysentery, para-dysentery and *Salmonella* groups. In addition to the poor absorption of this compound from the gut of rabbits, its antibacterial activity was found by them to be greater than that of sulphanilyl-guanidine. Since no detailed work on the toxicity, absorption and blood concentration of sulphanilyl-benzamide appears to have been done certain investigations have been undertaken with samples of this compound (M.P. 181°C.-182°C.) prepared in this laboratory. The results are described below.

EXPERIMENTAL.

Acute toxicity in mice.—Sulphanilyl-benzamide being very insoluble in water an emulsion in olive oil was used for testing its toxicity by intraperitoneal injection. Tests for toxicity by intravenous injection were made with a solution of the compound prepared by the addition of a minimum quantity of caustic soda and adjusting the pH between 8 and 9. The dosage used and the number and weight of the animals employed are given in Table I :—

TABLE I.

Showing the result of the toxicity tests (in mice) of sulphanilyl-benzamide.

Mode of administration.	Number of mice.	Average weight in g.	Dose, mg./g. body-weight.	Mortality in 96 hours.	Symptoms.	REMARKS.
Intravenous	12	18	0.25	0/12	Slight excitement.	
"	12	18	0.5	0/12	" "	
"	8	18	1.0	0/8	" "	
"	10	20	1.5	2/10	" "	
Intraperitoneal	10	20	1.0	0/10	Nil.	Higher dosage was not tried as a stronger emulsion than 8 per cent could not be accurately administered.
"	8	21	2.0	1/8	Nil.	

Concentration in blood.—For the estimation of blood concentration the technique employed was essentially the same as that followed by one of us in a previous study of soluble sulphanilamide derivatives (Bose, 1942). The drug was given orally to mice and rabbits as a 4 per cent emulsion in olive oil. For mice, a 2·5 per cent watery solution was also used when working with the lower dosage of 0·1 mg./g. The modified colorimetric method described by Marshall and Litchfield (1938) was employed for the estimations, using dimethyl- α -naphthylamine as the coupling reagent. For comparative purposes the experiments in mice were repeated with sulphanilamide using equal amounts and molecular proportions equivalent to those used in the case of sulphanilyl-benzamide. The results are given in Table II:—

TABLE II.
Showing the concentration of drug in blood.

Animal used.	Drug.	Method of administration.	Dose, mg./g. body-weight.	Hour of sample.	CONCENTRATION OF DRUG PER 100 C.C. OF BLOOD.		Percentage of conjugation.
					Free, mg.	Total, mg.	
Mice ..	Sulphanilyl-benzamide	Oral	0·5 in emulsion	$\frac{1}{2}$	16·7	20·0	16·6
				1	20·0	22·2	9·9
				2	25·0	33·3	24·9
				3	19·6	22·2	13·2
				4	13·6	15·1	10·0
Mice ..	Sulphanilyl-benzamide	Oral	0·25	$\frac{1}{2}$	10·7	10·7	<i>Nil.</i>
				1	24·2	21·4	<i>Nil.</i>
				2	11·3	12·0	5·8
				3	10·4	11·9	12·6
				4	7·7	8·8	12·5
Mice ..	Sulphanilyl-benzamide	Oral	0·10	1	13·4	13·4	<i>Nil.</i>
				2	6·7	7·5	1·7
				3	5·6	6·4	12·5
				4	4·5	5·4	16·6
Mice ..	Sulphanilamide	Oral	*0·31	$\frac{1}{2}$	22·8	22·2	<i>Nil.</i>
				1	10·5	11·0	3·8
				1½	8·8	9·7	8·7
				2	7·9	9·7	18·0
				3	6·8	8·4	19·0
Mice ..	Sulphanilamide	Oral	0·10	1	6·4	7·4	13·5
				2	4·3	5·2	17·3
				3	3·2	4·3	25·6
				4	3·4	4·7	27·7
Rabbits..	Sulphanilyl-benzamide	Oral	0·50	$\frac{1}{2}$	10·8	10·7	<i>Nil.</i>
				1	6·1	6·6	7·9
				2	7·5	8·1	6·6
				3	7·9	8·0	0·9

* Dose equivalent to 0·5 mg. sulphanilyl-benzamide.

Absorption from gastro-intestinal tract.—In order to ascertain the amount and the rapidity of absorption of sulphanilyl-benzamide from the gut, five mice of similar weight were fed with a dose of 0·25 mg. of the drug per gramme body-weight and the pooled contents of the stomach and the intestines examined separately at intervals of one and four hours. Assay

was done according to the usual procedure after the contents had been macerated with sand and distilled water. The results are given in Table III :—

TABLE III.

Showing the amount of sulphanilyl-benzamide recovered from the gastro-intestinal tract of mice.

Average weight in g.	Total amount of drug fed, mg.	Hour of sample.	AMOUNT IN MG. RECOVERED FROM	
			Stomach.	Intestine.
20	5	1	1.5	0.6
20	5	4	0.4	Trace.

SUMMARY AND CONCLUSIONS:

The results obtained (*vide* Table I) indicate that sulphanilyl-benzamide is free from undue toxicity, and that it appears to be better tolerated than sulphanilyl-guanidine (*cf.* Marshall *et al.*, 1940). Absorption from the gastro-intestinal tract of mice seems to be fairly rapid as shown by the disappearance of the drug within four hours (Table III). In mice sulphanilyl-benzamide reaches a moderately high concentration in the blood a half to one hour after administration, very similar to that produced by equimolecular proportions of sulphanilamide at the same interval after administration. The concentration of sulphanilyl-benzamide, however, tends to be maintained at a steadier level than is the case with sulphanilamide. From column 7, Table II, it is evident that the percentage of conjugation in the case of sulphanilyl-benzamide is much lower than that in the case of sulphanilamide, when the drugs are administered in low dosage (0.1 mg./g.). In rabbits it was found that the drug did not reach as high a concentration in the blood as in mice, but the figure obtained indicated a fair degree of absorption:

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USE OF TRICHLORETHYLENE AS AN ANÆSTHETIC AGENT: AN EXPERIMENTAL STUDY ON LABORATORY ANIMALS.

BY

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TRICHLORETHYLENE, $\text{CCl}_2 : \text{CHCl}$, first described in 1864, has mostly been used for dry-cleaning clothes, de-greasing metals and de-waxing lubricating oils. Interest in the medicinal use of this substance was created by the report of Plessner (1916) who first observed the elective action of this drug on the trigeminal nerve and recommended its use in trigeminal neuralgia. A number of workers (Joachimoglu, 1921; Herzberg, 1934; Jackson, 1934; Krantz *et al.*, 1935) subsequently studied the pharmacological and toxicological effect of this substance. An analysis of these studies has been published by the Council on Pharmacy and Chemistry of the American Medical Association (1936). On the basis of the data then available, the Council decided that it was not desirable to accept trichlorethylene as a general anæsthetic until further clinical and experimental work had been carried out. More recently, 'Trilene' (a stabilized form of the trichlorethylene) has been used in surgical anæsthesia with apparent success by Hewer (1941, 1942), Elam (1942), Gordon and Shackleton (1943) and Haworth and Duff (1943).

An inquiry as to its suitability for use in India led us to investigate this problem. The present work thus comprises an experimental study of the anæsthetic and toxic effects of trichlorethylene in comparison with chloroform and ether.

EXPERIMENTAL.

1. *Materials and methods.*—Originally the experiments were intended to be carried out with 'Trilene', an imported brand of trichlorethylene purified and stabilized with 0.01 per cent thymol. As this substance was not available in sufficient quantities, the work was continued with trichlorethylene purified and stabilized in Indian laboratories. In all, three samples of trichlorethylene have been used: (a) one specimen of trichlorethylene purified in India from the crude product, (b) one specimen further purified* from (a) and stabilized with alcohol, (c) a sample of 'Trilene' supplied by the Imperial Chemical (Pharmaceuticals), Ltd., England.

The anæsthetic and toxic effects of these three samples of trichlorethylene were studied on mice, guinea-pigs and rabbits. For mice both 'closed' and 'open' methods of anæsthesia were used but for guinea-pigs and rabbits the 'open' method only was employed. In the 'closed' method, the animals were kept in an air-tight cone of 180 c.c. air-space and the anæsthetic administered through the narrow open end of the cone. For the 'open' method

* The 'purified' trichlorethylene agreed with the following chemical specifications:—

- (i) Sp. gr. at 20°C. between 1.461 and 1.468; distillation range between 86°C. and 88°C.
- (ii) Residue on evaporation—not more than 0.01 per cent.
- (iii) Acidity—not more than 0.002 per cent as HCl.
- (iv) Free chlorine—nil.

a suitable mask was used in the usual manner for inducing general anaesthesia. The anaesthetics were given from a tuberculin syringe at the rate of one drop every 20 seconds. Guinea-pigs anaesthetized with ether required 6 drops per minute. Rabbits showed a peculiar tendency to hold their breath in the first and second stages of anaesthesia and to breathe rapidly only when the mask was lifted for re-charging. The mask was re-charged every minute with four successive drops of 'Trilene', trichlorethylene, or chloroform and with ten drops of ether.

2. *Findings*—(a). *The mean anaesthetic and lethal doses with their standard deviation figures are given in Table I.*

From an analysis of Table I it will be observed that in all three species of animals, chloroform showed the greatest anaesthetic effect and ether the least. The anaesthetic and lethal doses of trichlorethylene were found to be similar to those of 'Trilene' and both of them resembled chloroform more than ether in their mode of action. Induction of anaesthesia with trichlorethylene was comparatively easy but, unlike chloroform, initial fatal vagal-inhibition seldom occurred and post-anaesthetic toxic manifestations were infrequent. Trichlorethylene did not produce muscular relaxation of the same degree as was obtained with chloroform or ether. With the former, blinking of the eyelids, nodding movement of the head and even convulsions were not infrequent. Stiffness of the extremities and the phenomenon of tail-raising, present in a certain proportion of animals, showed an incomplete resolution of spinal centres as in the case of nitrous-oxide anaesthesia.

A comparison of the mean anaesthetic and lethal dose figures in Table I indicates that the margin of safety, as judged by the ratio of the two doses, is greatest with ether (from 2.5 to 3 in mice and about 4 in guinea-pigs). With chloroform and trichlorethylene this ratio was about 2 in mice ('open' method), and 2 and 3 respectively in guinea-pigs. It would thus appear that the margin of safety is slightly less with chloroform than with trichlorethylene.

Elimination of the anaesthetic, as judged by the reappearance of corneal reflexes and the recovery to the normal state, seemed to be quicker with chloroform than with 'Trilene' or trichlorethylene; with the two latter there were also localized and generalized spasms while recovering from the anaesthesia.

Under 'Trilene' and trichlorethylene the pulse was first irregular and fibrillating but later slowed down. The respiration was, however, accelerated as by ether. The findings of Jackson (*loc. cit.*) were thus corroborated. In the case of 'Trilene' and trichlorethylene, death occurred from primary respiratory failure preceded by cyanosis, while with chloroform, cardiac failure was the primary cause of death. Respiratory failure was much more gradual with iether than with 'Trilene' and trichlorethylene, and the heart appeared to be better sustained.

Trichlorethylene vapour did not appear to have as much irritant effect on the respiratory passages as ether. No excessive salivation or secretion of mucus was noticed. The vapour of 'Trilene' and trichlorethylene, however, proved to be irritant to the eyes and a good deal of lacrymation (also observed by Jackson, *loc. cit.*) and congestion of the conjunctiva were produced though no true conjunctivitis was observed.

(b) *Chronic toxic effects.*—These were studied on mice and guinea-pigs with increasing doses of the anaesthetics starting with the mean anaesthetic dose and going up to the mean lethal doses for each species of animals. In the case of mice the animals were prepared by the 'closed' method of administration and killed with the same substance on the 8th day. In the case of guinea-pigs, the 'open' method was used and the animals were killed on the 10th day. In Table II, the various morphological changes in various organs of guinea-pigs have been summarized and the total dose and the total duration of anaesthesia indicated. The microscopic appearances of the lung, liver and kidney of guinea-pigs treated with trichlorethylene and chloroform are shown in Plate I (figs. 1, 2, 3, 4). For brevity the findings in mice have not been included, but these conform with those in guinea-pigs.



Fig. 1. Effect of trichlorethylene on lungs (low magnification). Patchy consolidation, emphysematous areas and desquamation of bronchiolar epithelium indicated by arrows.

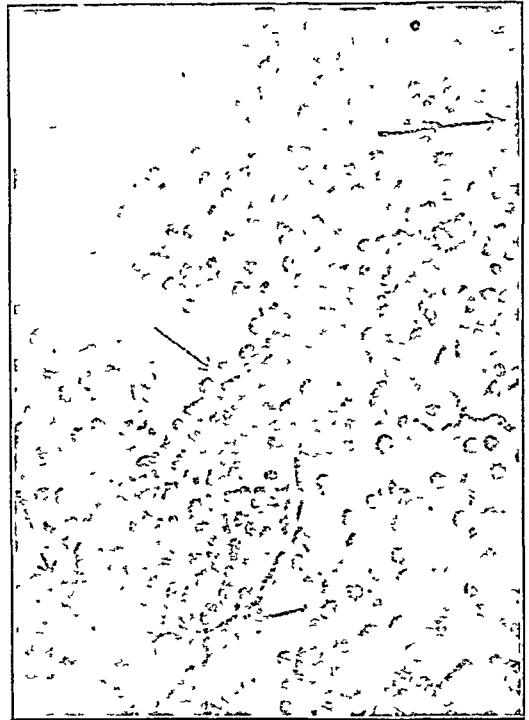


Fig. 2. Effect of trichlorethylene on kidneys (high magnification). Glomerular hemorrhage indicated by arrow marks and swelling of tubular epithelium.

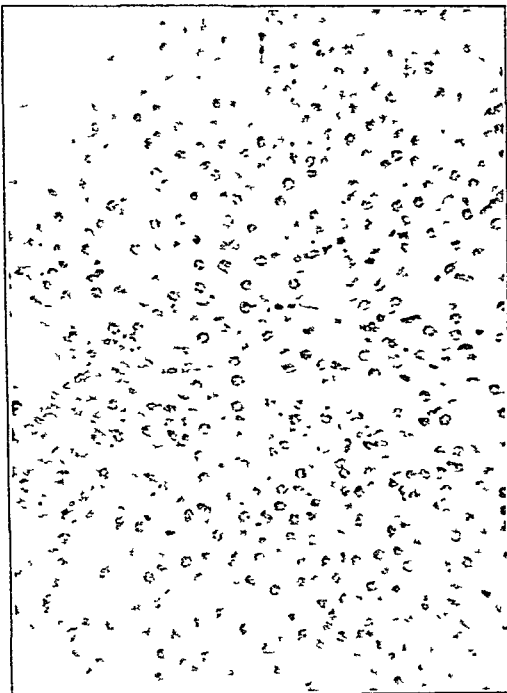


Fig. 3. Effect of trichlorethylene on liver (low magnification). Little degenerative and fatty changes.

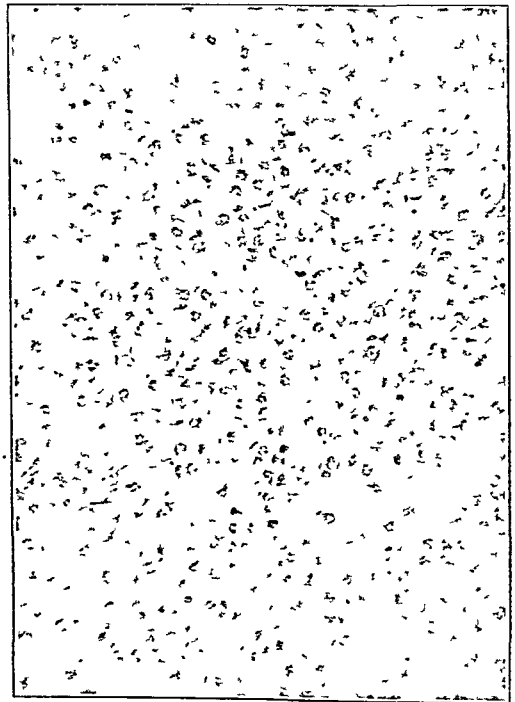


Fig. 4. Effect of chloroform on liver (low magnification). Swelling and vacuolation of liver cells and fatty degeneration.

TABLE I.

Showing the mean anaesthetic and lethal doses in 100 mice, 60 guinea-pigs and 27 rabbits.

Name of anaesthetics.	MICE.				GUINEA-PIGS.				RABBITS.	
	'CLOSED' METHOD.		Corrected concentration.	Mean lethal dose, c.c.	'OPEN' METHOD.		Mean anaesthetic dose, c.c.	Mean lethal dose, c.c.	Mean anaesthetic dose, c.c.	Mean lethal dose, c.c.
	Mean anaesthetic dose, c.c.	Corrected concentration.			Mean anaesthetic dose, c.c.	Mean lethal dose, c.c.				
'Trilene' [Imp. Chem. (Pharmaceuticals), Ltd.],	$\left\{ \begin{array}{l} 0.071 \\ \pm 0.007 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 6,576 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 3,946 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.130 \\ \pm 0.010 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.110 \\ \pm 0.013 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.190 \\ \pm 0.022 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.218 \\ \pm 0.011 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.590 \\ \pm 0.013 \end{array} \right\}$	$\left\{ \begin{array}{l} \cdot \cdot \\ \cdot \cdot \end{array} \right\}$	$\left\{ \begin{array}{l} \cdot \cdot \\ \cdot \cdot \end{array} \right\}$
Trichlorethylene (batch 1)	$\left\{ \begin{array}{l} 0.072 \\ \pm 0.013 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 6,576 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 3,946 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.130 \\ \pm 0.010 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.110 \\ \pm 0.015 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.205 \\ \pm 0.005 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.230 \\ \pm 0.021 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.590 \\ \pm 0.028 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.450 \\ \pm 0.033 \end{array} \right\}$	$\left\{ \begin{array}{l} 1.570 \\ \pm 0.075 \end{array} \right\}$
Trichlorethylene (batch 2)	$\left\{ \begin{array}{l} 0.070 \\ \pm 0.004 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 6,576 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 4,003 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.140 \\ \pm 0.018 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.113 \\ \pm 0.007 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.230 \\ \pm 0.017 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.210 \\ \pm 0.008 \end{array} \right\}$	$\left\{ \begin{array}{l} \cdot \cdot \\ \cdot \cdot \end{array} \right\}$	$\left\{ \begin{array}{l} \cdot \cdot \\ \cdot \cdot \end{array} \right\}$	$\left\{ \begin{array}{l} \cdot \cdot \\ \cdot \cdot \end{array} \right\}$
Chloroform (Beng. Chem. Pharmaceut. Works, Cal.).	$\left\{ \begin{array}{l} 0.049 \\ \pm 0.004 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 7,500 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 3,816 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.123 \\ \pm 0.020 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.070 \\ \pm 0.017 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.145 \\ \pm 0.009 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.160 \\ \pm 0.015 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.330 \\ \pm 0.020 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.270 \\ \pm 0.026 \end{array} \right\}$	$\left\{ \begin{array}{l} 1.070 \\ \pm 0.075 \end{array} \right\}$
Ether (Beng. Chem. Pharmaceut. Works, Cal.).	$\left\{ \begin{array}{l} 0.210 \\ \pm 0.022 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 2,642 \end{array} \right\}$	$\left\{ \begin{array}{l} 1 \text{ in } 1,438 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.500 \\ \pm 0.010 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.220 \\ \pm 0.034 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.590 \\ \pm 0.026 \end{array} \right\}$	$\left\{ \begin{array}{l} 0.760 \\ \pm 0.088 \end{array} \right\}$	$\left\{ \begin{array}{l} 2.820 \\ \pm 0.280 \end{array} \right\}$	$\left\{ \begin{array}{l} 2.450 \\ \pm 0.050 \end{array} \right\}$	$\left\{ \begin{array}{l} \cdot \cdot \\ \cdot \cdot \end{array} \right\}$

TABLE II.

Showing toxic effect of 'Trilene', trichlorethylene, chloroform and ether on heart, lungs, liver and kidneys of guinea-pigs.

Name of anaesthetics.	Total dose in 10 days, c.c.	TOTAL PERIOD OF ANÆSTHESIA.		Heart.	Lungs.	Liver.	Kidneys.
		Hours.	Mins.				
'Trilene' [Imp. Chem. (Pharmaceuticals), Ltd.].	4.29	3	10	Practically normal	Patchy areas of consolidation and focal hemorrhage. Altered consolidation and emphysema.	(Cloudy swelling but no fatty change.	Signs of acute toxic nephritis with hemorrhage in the malpighian bodies and swelling and degenerative changes in tubular epithelium.
Trichlorethylene (batch 1)	4.29	3	10	Definite signs of cloudy swelling and fragmentation of certain bundles.	Greater consolidation than in the former; desquamation of epithelium in certain bronchioles.	Fatty infiltration of hepatic cells with well-marked congestion and hemorrhage between the cords of liver cells.	Similar changes but more hemorrhage in glomeruli and between tubules, tubular epithelium less involved than in the former case.
Trichlorethylene (batch 2)	4.29	3	10	Certain amount of swelling of bundles.	More hemorrhage and consolidation than in 'Trilene' but less than in trichlorethylene (batch 1).	(Congestion in central veins; little degenerative or fatty changes.	" " "
Chloroform (Beng. Chem. Pharmaceut. Works, Cal.).	2.45	2	55	Changes similar to trichlorethylene (batch 1).	Very little hemorrhage but patchy bronchopneumonic consolidation alternating with emphysematous areas.	Well-marked swelling and vacuolation of liver cells; marked degenerative changes.	Hemorrhages in glomerular and tubular areas; destruction of tubular cells; hæmorrhage greater than in other cases.
Ether (Beng. Pharmaceut. Works, Cal.).	18.76	3	42	Normal	Patchy consolidation present but little hæmorrhage or emphysema.	No fatty change but signs of mild degree of cloudy swelling.	Swelling of glomeruli but no hemorrhage; swelling of tubular epithelium.

It will be seen from Table II that, in toxicity, chloroform ranks highest and ether lowest amongst the three anaesthetics studied. Contrary to the findings of Joachimoglu, Herzberg and Krantz (*loc. cit.*), 'Trilene' and trichlorethylene were found to be toxic to the kidneys and lungs and produced broncho-pneumonic and acute toxic nephritic changes. This is in accord with the findings of Lande *et al.* (1939) who also observed pathological changes in the kidney following prolonged administration of trichlorethylene.

Experiments with graded doses of purified trichlorethylene showed that 'Trilene' was the least toxic of the three samples studied. Sample 1 of trichlorethylene was found to be slightly more toxic than sample 2. The latter sample, under identical conditions of animal experiments, showed 15 to 20 per cent greater toxicity than 'Trilene'. This difference may not be of any serious consequence, as such high dosage is not likely to be used in surgical anaesthesia. Animals submitted to 50 per cent of the above dosage failed to show any appreciable pathological changes.

CONCLUSIONS.

1. A comparative study of the anaesthetic and toxic action of 'Trilene', trichlorethylene, chloroform and ether has been made on mice, guinea-pigs and rabbits.

2. In anaesthetic properties, 'Trilene' and trichlorethylene showed certain characteristics common to both chloroform and ether, but muscular relaxation was almost as imperfect as with nitrous oxide.

3. Both 'Trilene' and trichlorethylene are less toxic than chloroform, but ether proved to be safer than either of them. The anaesthetic dose of 'Trilene' and trichlorethylene is slightly greater than that of chloroform but is much less than that of ether.

4. Favourable features of trichlorethylene anaesthesia are its rapidity of action, its analgesic effect and relative absence of early fatal accidents. Its chief disadvantage is its failure to produce complete muscular relaxation and when this is required, resort must be had to supplementary ether anaesthesia. In view of observed toxic effects on the kidney and lung after administration of heavy doses of trichlorethylene, it would be injudicious in the present state of knowledge to use trichlorethylene in cases where prolonged anaesthesia is required.

5. No difficulty was experienced by the authors in inducing anaesthesia in animals without the use of special apparatus.

ACKNOWLEDGMENTS.

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OBSERVATIONS ON MALIGNANT DISEASE IN CEYLON BASED ON A STUDY OF TWO THOUSAND TWO HUNDRED AND NINETY-FIVE BIOPSIES OF MALIGNANT TUMOURS.

BY

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NATH AND GREWAL (1935, 1937, 1939) have shown in their exhaustive study that malignant disease is not uncommon in India. Although their data were based on clinical, autopsy and biopsy records, they have stated that these offered only a very rough measure of the cancer incidence in India. In Ceylon too, an accurate estimate of the incidence of malignant disease is impossible, but a rough idea regarding its prevalence could be obtained by a perusal of Table I which gives the number of admissions and deaths from malignant disease at the General Hospital, Colombo, during the four-year period 1939 to 1942. Malignant disease accounted for 4,027 admissions which is nearly 1/30 of the total admissions.

TABLE I.

Admissions and deaths from malignant disease at the General Hospital, Colombo.

	1939.		1940.		1941.		1942.		Total admissions.	Total deaths.
	Admissions.	Deaths.	Admissions.	Deaths.	Admissions.	Deaths.	Admissions.	Deaths.		
Buccal cavity ..	507	15	445	16	496	16	460	17	1,908	64
Stomach and liver ..	49	12	37	9	48	16	76	23	210	60
Peritoneum, intestine, rectum.	61	5	43	5	67	12	60	19	231	41
Female genital organs	230	18	154	12	200	16	124	15	708	61
Breast	49	5	57	4	78	7	49	5	233	21
Skin	39	4	25	3	39	3	31	3	134	13
Organs not specified ..	172	12	111	3	136	16	184	20	603	51
TOTALS ..	1,107	71	872	52	1,064	86	984	102	4,027	311

Table II shows some of the main causes of morbidity during the same period and it will be seen that malignant disease exceeded the morbidity caused by typhoid fever and was responsible almost to the same degree as tuberculosis, influenza and pneumonia in the creation of a morbid state:—

TABLE II.

Main causes of morbidity.

		1930.		1940.		1941.		1942.		Total admissions.	Total deaths.
		Admissions.	Deaths.	Admissions.	Deaths.	Admissions.	Deaths.	Admissions.	Deaths.		
Malaria	4,036	157	3,715	152	2,996	84	1,373	39	12,120	432
Pneumonia	1,568	284	1,762	203	1,155	182	675	98	5,160	767
Typhoid	944	206	727	150	896	109	570	112	3,146	667
Influenza	1,140	20	1,507	7	1,211	13	764	6	4,622	46
Tuberculosis	861	194	1,012	190	1,260	291	988	11	4,121	686
Chronic ulcers	864	10	518	6	567	19	520	11	2,469	46
TOTALS	33,295	2,655	36,182	2,483	32,867	2,502	22,463	1,628	124,807	9,268

As a preliminary investigation regarding malignant disease in Ceylon, an attempt has been made to make an analysis of the malignant tumours sent to the Department of Pathology, University of Ceylon, for histological examination from various hospitals in the island, during the seven-year period 1936 to 1942 (biopsies from all the hospitals in Ceylon are examined in this department). The total number of specimens examined histologically during the period was 10,880. Most of these were removed at operations. Very few had been obtained post-mortem. Of the total number of specimens, malignant tumours amounted to 2,295 or 21.3 per cent of the specimens examined.

Table III gives the types of malignant tumours studied. Primary carcinomata constituted 79.1 per cent. The special tumours in the table includes some rare neoplasms and on account of their rarity they have been classified into a separate group.

TABLE III.

Types of malignant tumours.

Primary carcinomata	1,815	(79.1 per cent)
Secondary carcinomata	95	(4.1 " ")
Primary sarcomata	188	(8.2 " ")
Special tumours	22	(1.0 " ")
Hæmangio-endotheliomata	10	(0.4 " ")
Malignant endotheliomata	35	(1.5 " ")
Malignant melanomas	41	(1.8 " ")
Lymphadenomas	32	(1.4 " ")
Lymphosarcomas	18	(0.8 " ")
Lympho-epitheliomas	5	(0.2 " ")
Potential cancers 23 ; pre-cancerous 11	34	(1.5 " ")
			2,295	

Although there is much controversy regarding the nature of lymphadenoma, we have for the purpose of this study looked upon it as a malignant tumour.

Malignant melanoma has been included in a separate category, as its origin is still controversial and as this group of tumours shows a high incidence in Ceylon.

Of the 95 secondary carcinomas, 75 occurred in lymphatic glands, 12 in the omentum, 7 in the liver and one in a hernial sack. As the primary site of these neoplasms are unknown these will not be considered any further.

It is of interest to compare the incidence of malignant tumours based on histological studies carried out by Nath and Grewal (*loc. cit.*) in India. Table IV, which gives the incidence of malignant tumours based on the examination of biopsy specimens, is comparable with our study. It will be seen that there is not much variation in the incidence of malignant disease in different parts of India and in Ceylon. The incidence here appears to be slightly higher than that of Madras, Bombay and Bengal and slightly lower than that of Rangoon.

TABLE IV.

Incidence of malignant tumours in biopsy material in India and Ceylon.

Place			Number of specimens studied.	Number of malignant tumours.	Percentage incidence of malignant tumours.
Madras	11,801	2,236	18.9
Rangoon	2,814	741	26.3
Bombay	9,855	1,848	18.8
Bengal	6,143	1,136	18.5
Ceylon	10,800	2,295	21.3

The relationship of the sarcomata to the carcinomata based on biopsy records in different parts of India and in Ceylon are given in Table V. The incidence of sarcoma in Ceylon appears to be very much lower than that in India. The incidence of sarcoma according to our study is 8.2 per cent and corresponds to standards in the United States of America, which according to Hoffman (1925), varies from 8 to 10 per cent. The relatively lower incidence of sarcoma in Ceylon may possibly be due to a lack of ætiological factors, such as infective granulomata, which are more prevalent in India and which are definitely recognized to predispose to sarcoma.

TABLE V.

Relationship of sarcomata to carcinomata in biopsy material in India and Ceylon.

			Sarcoma.	Carcinoma.	Ratio of sarcoma to carcinoma.
Madras and Burma	552	2,171	1 : 4
Central Provinces	756	2,507	1 : 3
Delhi, United Provinces, Bihar & Orissa..			1,117	2,531	1 : 2
Ceylon	188	1,815	1 : 10

THE PRIMARY CARCINOMATA.

Table VI shows the primary site of the growth, the sex and racial distribution. The 1,815 carcinomata arose in 31 different sites. The regional distribution according to sex is shown

in Table VI-a. 44.2 per cent of the tumours arose in the generative organs in both sexes. In nearly half the number of females and one-third the number of males the primary growth was removed from the genitals, which included the penis, prostate and testicle in the male and uterus, ovary, fallopian tube, vulva and vagina in the female. The cervix alone was the primary site in nearly one-third of the female cancers and the penis in more than a third of the male cancers. Cervical and penile cancer does not bear the same degree of prevalence in European countries (British Empire Cancer Campaign, 1941; Hoffman, 1940). The other sites in order of frequency were as follows: buccal cavity, skin, breast, gastro-intestinal tract including liver, gall-bladder and pancreas, the nose and larynx, the thyroid gland, eyelids, conjunctiva and cornea, urinary tract, parotid gland, pharynx, and the ear.

TABLE VI.

Sites.	MALES.								FEMALES.								Total males and females.
	Sinhalese.	Tamils.	Burghers.	Moors.	Malays.	Europeans.	Indians.	Total.	Sinhalese.	Tamils.	Burghers.	Moors.	Malays.	Europeans.	Indians.	Total.	
1. Cervix	266	28	17	5	316	316
2. Buccal cavity ..	121	50	5	16	1	2	1	196	53	18	2	4	..	1	..	78	274
3. Penis	209	35	2	2	248	248
4. Breast	3	2	5	129	13	17	7	1	3	..	170	175
5. Skin	128	29	8	6	1	3	..	175	55	9	4	3	71	246
6. Prostate	37	8	3	1	1	50	50
7. Testis	11	4	..	1	19	19
8. Ovary	59	6	3	2	..	70	70
9. Uterus—body, chorion epithelioma }	26	1	4	1	..	32	32
10. Fallopian tube	27	2	4	1	..	1	..	35	35
11. Vulva and vagina	1	1	1
12. Thyroid	12	1	1	14	22	1	1	..	24	32
13. Parotid	6	4	..	1	11	9	2	1	12	23
14. Oesophagus	4	4	5	5	9
15. Stomach	3	11	4	..	2	1	3	7
16. Small intestine ..	1	1	1	1	2
17. Cæcum	2	1	1	1	..	5	2	5
18. Large intestine ..	4	..	1	5	2	4	7
19. Rectum	11	2	1	1	15	1	..	3	4	19
20. Pharynx	4	1	2	1	8	1	1	9
21. Tonsil	4	1	5	2	4	9
22. Nose	8	6	1	15	9	1	10	25
23. Larynx and post- ericoid region. ..	11	3	..	1	15	1	1	1	3	18
24. Liver	4	2	6	3	3	9
25. Gall-bladder	1	1	1
26. Pancreas	1	1	1	1	2
27. Kidney	4	2	1	7	3	3	10
28. Urinary bladder ..	4	..	2	1	7	7
29. Urethra	1	1	6	1	7	8
30. Conjunctivæ, eyelids } ..	12	1	1	2	16	2	2	18
31. Ear (polypi)	1	1	5	1	1	7	19
32. Unknown	34	13	3	50	16	3	1	1	20
TOTALS	651	170	32	33	2	6	3	897	733	94	59	22	1	9	..	918	1,815

TABLE VI-a.

Regional distribution of the 1,815 carcinomata.

	Males.	Females.	Total.	Percentage distribution.
Generative organs	317	486	803	44.2
Buccal cavity including tongue ..	196	78	274	15.1
Skin	175	71	246	13.6
Breast	5	170	175	9.6
Gastro-intestinal tract including liver, gall-bladder, pancreas.	42	19	61	3.4
Upper air passages (nose and larynx) ..	30	13	43	2.4
Endocrine glands (thyroid) ..	14	24	38	2.1
Eye (eyelids, conjunctivæ, cornea) ..	28	9	37	2.0
Urinary tract	15	10	25	1.4
Parotid gland	11	12	23	1.3
Pharynx and tonsil	13	5	18	1.0
Ear	1	1	2	.1
Unknown site	50	20	70	3.9
TOTALS ..	897	918	1,815	

Table VII gives the histological type and sex distribution of the primary carcinomata. The predominant type was the squamous variety, 65.8 per cent being squamous-cell carcinomata. The squamous cell is widely distributed under normal conditions and pathologically it occurs as the result of metaplastic conversion of other types of epithelium, due to chronic inflammatory processes and nutritional defects. The predominance of the squamous variety over others is therefore not unusual.

TABLE VII.

Histological classification of the carcinomata.

	Males.	Females.	TOTAL.
Squamous carcinoma	684	511	1,195
Adenocarcinoma	129	161	290
Carcinoma simplex	5	137	142
Encephaloid (medullary) carcinoma	8	8
Scirrhus carcinoma	18	18
Basal cell carcinoma (rodent ulcer) ..	14	5	19
Baso-squamous carcinoma	10	5	15
Transitional cell carcinoma	7	..	7
Chorion epithelioma	35	35
Hypernephroma	5	2	7
Hepatoma (liver-cell carcinoma) ..	5	2	7
Cholangioma (bile-duct carcinoma) ..	1	1	2
Teratoma	5	6	11
Seminoma (dysgerminoma)	14	6	20
Mucoid carcinoma	2	2
Granulosa cell tumour	2	2
Luteinoma	1	1
Lympho-epithelioma	2	3	5
Undifferentiated carcinoma	16	13	29
TOTALS ..	897	918	1,815

Sex incidence.—All workers are agreed that cancer occurs more frequently in women than in men. Kettle (1925) for instance mentions that in the years 1901 to 1903, 33,788 men and 50,660 women died of cancer. Although our figures support this well-established fact, yet the difference in the cancer incidence between males and females is very small. In other countries, the greater incidence in female cancers is attributed to the frequency with which the female generative organs, cervix, uterus, ovaries, and the breast are liable to undergo malignant change. In Ceylon, however, the relative proportions of male and female cancer are not the same as in other countries, on account of the remarkably high incidence of penile cancer.

Racial incidence.—The population of Ceylon is composed of a mixture of races. The racial composition of the population shown in Table VIII is based on the 1931 Census and may be considered a fair indication of the actual composition during the period under review. If our figures are considered to reveal the actual incidence of cancer they show that only a small proportion of the Sinhalese, Tamils, Moors and Malays are afflicted. The figures, however, far from indicating the cancer incidence, only show the number of hospital admissions on whom biopsies have been done for diagnosis.

TABLE VIII.

Population and incidence of carcinoma in biopsy material.

			Total population.	Carcinoma.	Incidence per 1,000 of population.
Sinhalese	3,473,029	1,381	0.4
Tamils (India and Ceylon)	1,477,477	267	0.1
Burghers	32,315	91	2.8
Moors	321,913	55	0.1
Europeans	9,154	15	1.6
Malays	15,977	3	0.2

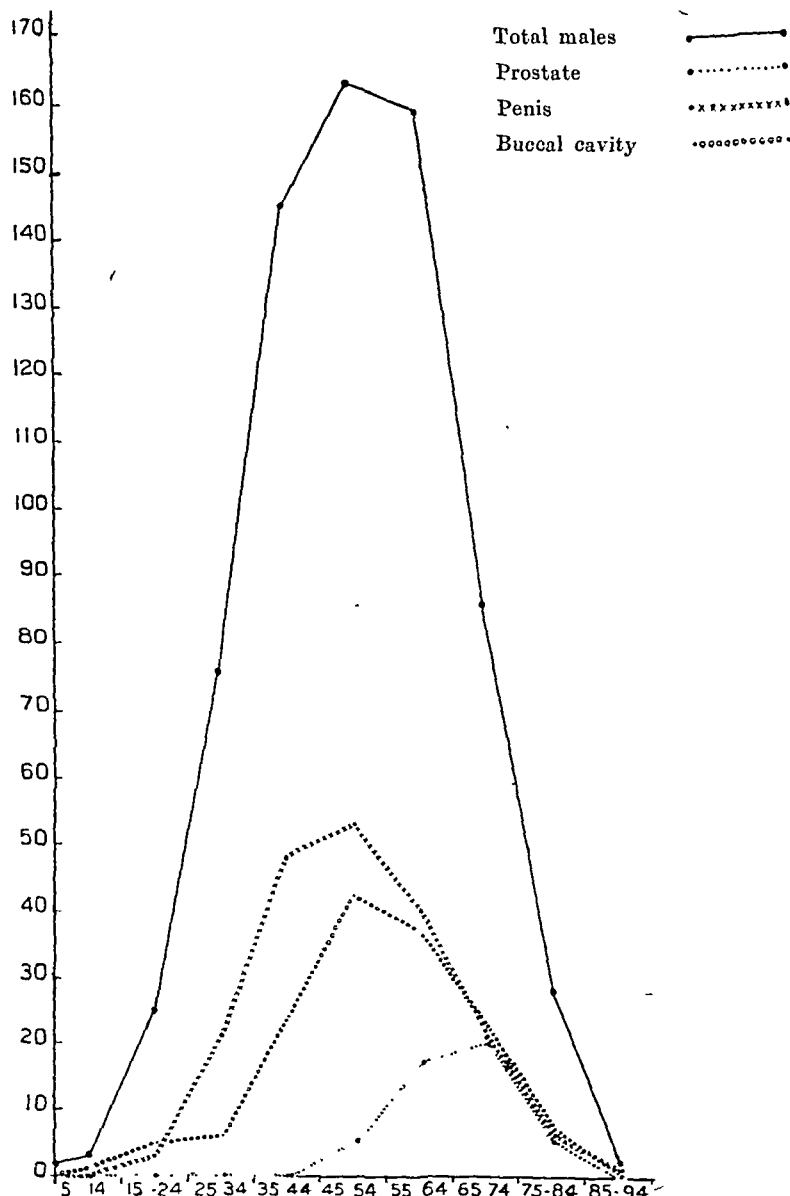
It cannot be argued from these figures that cancer is not so common in the Sinhalese, Tamils, Moors and Malays as in the European and the Burgher. It could, however, be reasonably concluded that the European and the Burgher seek hospital admission more frequently than the rest. This is probably due to the fact that their information and knowledge regarding such an insidious disease process like cancer hasten them to seek medical relief more frequently and much earlier than the other races.

Age incidence (vide Graphs 1 and 2).—The age was recorded in the case of 689 males and 795 females. The ages in the case of the former varied from 10 months to 94 years and in the latter from 3 years to 79 years. In both sexes the highest incidence of carcinoma was in the ten-year period, 45 to 54: 63.8 per cent of female cancers and 52.1 per cent of male cancers occurred in the period of life between 35 and 64 years. Unlike most of the European countries and the United States of America, cancer begins to show high incidence in the 3rd decade of life reaching its maximum in the 4th decade. The age period 45 to 54 not only showed a maximum incidence in all the primary cancers taken together but also coincided with the maximum incidence of carcinoma occurring at each primary site, with but a few exceptions. Thus, the highest incidence of carcinoma of the cervix, penis, breast and buccal cavity was in the same age-group, the exceptions being the ovary and chorion epithelioma, where the maximum incidence was at an earlier period in life, viz. 25 to 34, and the prostate and the uterine body in which carcinoma occurred later in life on 65 to 74 and 55 to 64 respectively.

Carcinoma occurring at certain primary sites deserve further consideration.

1. *Cervix*.—It was stated earlier that cancer of the generative organs was noted in nearly half the number of females. Of the 486 cases involving the genital tract 316 occurred in the cervix. Histologically it was possible to differentiate three types: (1) adenocarcinoma, (2) squamous carcinoma with spines or intercellular bridges with a tendency to form keratinized

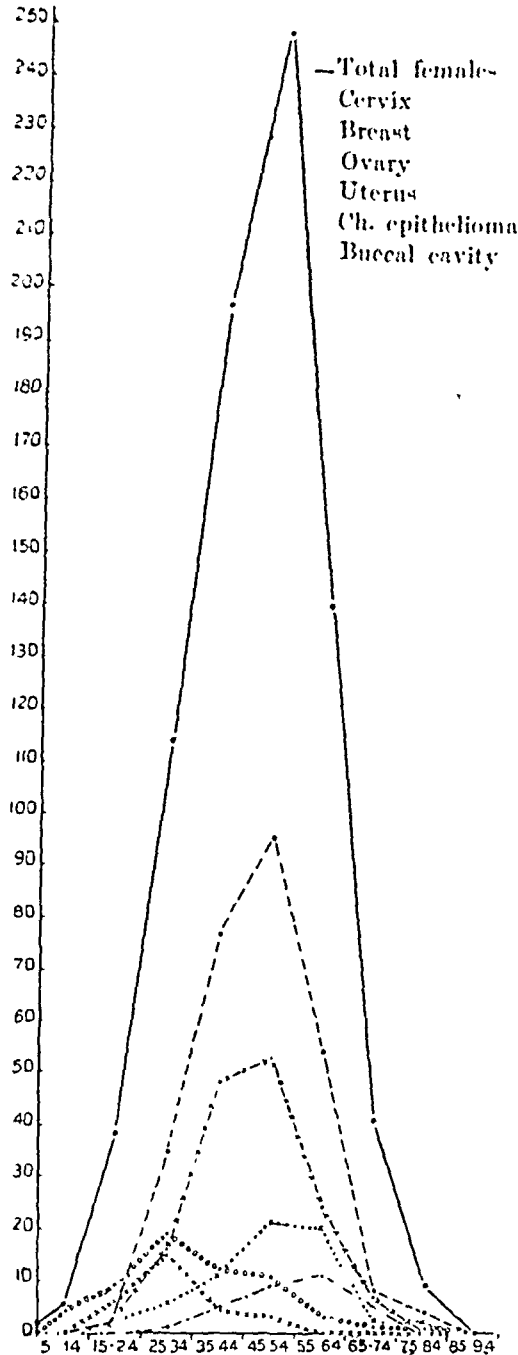
GRAPH 1.



'pearls', (3) a type which was not so well differentiated as (1) and (2) where the cells assumed a spindle or polygonal shape. Some of these showed giant cells. These on account of the marked anaplasia and the presence of mitoses suggested a very high grade of malignancy. Types (1) and (2) on account of the differentiation were regarded as the least malignant. There were only 17 adenocarcinomas, viz. type (1), the greater number of the cervical cancers being almost

equally composed of types (2) and (3). One hundred and fifty-five belonged to type (2) the squamous variety and 144 to type (3) the most malignant. The adenocarcinoma and the squamous-cell carcinoma originated from the cervical canal and the portio vaginalis respectively, but the undifferentiated type originated either from the portio vaginalis or from metaplasia of columnar epithelium of the cervical canal resulting from a long-standing cervicitis, which is very commonly found in women especially after parturition.

GRAPH 2.



Judging from this study it would appear that, in Ceylon, a large proportion of cervical cancers appears to be of a very high grade of malignancy. Treatment should therefore be commenced very early in order to prevent suffering caused by metastasis. Early treatment is only possible if these cases attend hospitals and clinics immediately after the onset of symptoms. But unfortunately it is common experience that such cases seek treatment at a time

when any form of therapy, beyond palliative measures is of no avail. [Attygalle (1937) mentions that in his series of 181 cases 98 belonged to stage iv of the international classification.] This fact is also amply emphasized in our study. Of the 316 cases of cervical cancer only in 8 was there evidence of early malignancy and only one was considered to be pre-cancerous.

The maximum age incidence of cervical cancer according to our figures was in the ten-year period 45 to 54 the period of the menopause, but quite a large number occurred in the reproductive period of life as well as the post-menopausal period. Only 3 cases occurred before the age of 24. The relationship of cervical cancer to parturition is well established in this country as well as in others (Stout, 1932). Certain ætiological factors, which predispose to cancer of the cervix, have been recognized. One of these which is widely prevalent in Ceylon is chronic endocervicitis, the chief cause of which is found in neglected injuries received during child birth. Therefore, it is evident that one of the most potent weapons for the control of this disease is prophylaxis. Post-natal examination is just as important as the ante-natal one. Leucorrhœa and hæmorrhage, especially post-menopausal, should not be disregarded. Serious notice should be taken of cervical erosions, retention cysts, polypi and lacerations which are known in some way or other to predispose to carcinoma. Many an early case of cancer will not fail to escape detection, if in addition to palpation of the cervix, it is also examined with the aid of a speculum.

2. *Cancer of the uterine body* was not so common as the cervix and occurred in the later years of life, the highest incidence being in the ten-year period 55 to 64. Although carcinoma of the uterine body is generally regarded as a disease of the post-menopausal period, our study indicates that in at least 6 women it arose between 25 and 44. The histological type was adenocarcinoma and only 5 out of the 32 cases showed early malignant changes.

3. *Chorion epithelioma*.—A malignant tumour which is more frequently met with in Ceylon than in other countries is the chorion epithelioma. Stout (*loc. cit.*) says that this is a relatively infrequent malignant tumour. Quinland and Cuff (1940) in a collection of 6,176 biopsy specimens and 813 autopsies in negroes does not mention a single case of chorion epithelioma. Hartz's (1940) analysis of 650 autopsies at Curaco, Netherlands, West Indies, does not refer to a single instance of this tumour. Yet in our series there were 35 chorion epitheliomas contributing to 7.2 per cent of the tumours of the genital tract.

The incidence of chorion epithelioma was higher than that of adenocarcinoma of the uterine body. This malignant tumour, which is invariably associated with a pregnancy, normal or abnormal, and follows hydatidiform mole in many instances, occurs in younger women during the reproductive period. The maximum age incidence in this series was in the age-group 25 to 34. Six cases were seen in women between 15 and 24 years of age. There appear to be two factors which favour the growth of these tumours in women of this country. Firstly, there is a greater tendency to hydatidiform degeneration of the chorionic villi. Thirty-two benign hydatidiform moles were seen during this period. Failure to evacuate these would have undoubtedly resulted in malignant change. Secondly, debilitating conditions like malaria and ankylostomiasis, the effects of successive pregnancies and general nutritional defects amongst the poorer classes appear to make the uterine muscle less resistant to the invasive action of the chorionic elements.

4. *Ovary*.—There were 70 malignant ovarian tumours in this series which formed 14.4 per cent of the malignant tumours of the female genital tract. They were more frequently met with in younger age-groups in contrast to other countries. According to Byron and Berkoff (1926) 60 per cent of the ovarian cancers lie between the ages of 40 to 60 years. In Stout's (*loc. cit.*) series 50 per cent occurred between 40 and 49 years. He states that only teratomata occur in the young, especially children. In our series the largest number, viz. 19, occurred in the younger age-group 24 to 34 years and in this group there were only 3 teratomata. Ten malignant ovarian tumours, which included only one teratoma, occurred in women under 20 years.

The types of ovarian tumours both innocent and malignant which were examined during this period is shown in Table IX:—

TABLE IX.

Types of ovarian tumours.

<i>Benign tumours—</i>						
All types of simple cysts (follicular, corpus luteum, endometrial) ..						66
Dermoid cysts	6
Pseudomucinous cystadenoma	101
Papillary cystadenoma	25
TOTAL ..						198
<i>Malignant tumours—</i>						
Malignant papillary cystadenomas	33
Pseudomucinous cystadenomas undergoing malignant change ..						9
Solid carcinoma	12
Teratoma	6
Dysgerminoma	6
Granulosa cell tumour	2
Unclassified	2
TOTAL ..						70
GRAND TOTAL ..						268

It will be seen that out of 268 cysts removed surgically 70 were malignant—a percentage of 26·1. Deaver (1928) reported 226 ovarian cysts operated upon of which only 23 or 10·2 per cent were malignant. Thus, there appears to be a greater frequency of malignant change in ovarian cysts in this country. This is probably once again due to delay in seeking medical advice.

A large proportion of primary malignant tumours were the papillary growths, more than half being malignant. The pseudomucinous cystadenoma less frequently appeared to undergo malignant change. However, in the control of ovarian cancer it is wise to regard all benign cystic neoplasms of the ovary—particularly the papillary cystadenoma—as definitely pre-cancerous lesions. Their early diagnosis and removal would reduce the incidence of malignant disease of the ovary.

5. *Buccal cavity.*—Cancer of the buccal cavity was only exceeded by that of the cervix. Our figures do not, however, give a correct idea of the prevalence of oral carcinoma, as a large number of cases that are admitted to hospitals are so advanced that a biopsy is rendered unnecessary for diagnosis. Figures obtained from the General Hospital show that out of 5,963 cancer admissions during the years 1937 to 1942, 2,496 or 42 per cent were carcinomas of the buccal cavity (tongue included). Even if subsequent admissions are ignored there appears to be a large number of the population suffering from buccal carcinoma. Paul's (1929) figures for 1928 also show a high incidence. Out of 415 cases of malignant disease admitted to the General Hospital in that year, 140 were cases of carcinoma of the buccal cavity (tongue included). Table X gives the sites from which biopsy specimens had been taken. The most frequent site of cancer of the buccal mucosa is the cheek and the gums. It is impossible in the later stage of the disease to decide whether the growth started as a carcinoma of the cheek or of the gums.

TABLE X.

Buccal carcinoma showing the sites affected.

				Males.	Females.	TOTAL.
Cheek	95	38	133
Tongue	36	11	47
Gums	23	20	43
Lip	30	6	36
Palate	12	3	15
TOTALS ..				196	78	274

The maximum age incidence of buccal cancer for both sexes is the ten-year period 1945 to 1954, which is slightly lower than that of European countries.

Though cancer of the mouth was more frequently seen in the 4th to the 7th decade yet cases occurred in every age-group from 5 to 44 years. Seven cases were seen before the age of 20. Almost all these were carcinomas of an advanced degree of malignancy, but a very small proportion, viz. 31 out of 274, showed histological appearance of early malignancy. The infrequency of early malignant changes in our biopsy specimens in contrast to the large number of specimens showing undoubted malignant changes, once again suggests the late arrival of cases to hospitals.

There appear to be two factors which predispose to buccal carcinoma: (1) betel-chewing with lime, (2) dental sepsis. Association of cancer with betel-chewing has been recognized by previous workers (White, 1913). Dental sepsis, which may partly be attributed to the habit of betel-chewing, is widely prevalent (Balendra, 1941), and clinical examination reveals that cases of cancer are associated with carious and misplaced teeth and pyorrhœa alveolaris. Such conditions constitute a fruitful source of chronic irritation, especially of the gums, cheek and the tongue, which promotes cancerous changes in these tissues.

6. *Penis*.—The primary site of carcinoma was noticed in the male generative organs in one-third the number of cases. Penile cancer was exceeded only by cervical and oral cancer. Paul (*loc. cit.*) found that carcinoma of the penis was the second commonest form of malignant disease in his series. Penile cancer is very much more widely prevalent in Ceylon though very rare in European countries and in the Americas, Squirru (1924, quoted by Stout, *loc. cit.*) encountered only 4 cases among 1,000 cancers in the Argentine. Shore (1928) records only 4 cancers among 1,000 miscellaneous cancers seen at St. Luke Hospital, New York. Statistics of the British Empire Cancer Campaign (*loc. cit.*) show 68 cancers of the penis in 15,068 cases of cancer. On the other hand several workers have shown the higher incidence of penile cancer in Asiatic countries, e.g. Cochin China, Dutch East Indies and Malays, Filipinos, and in India (de Vogel, Sitsen, Gnarzon and Rogers, 1932; Nath and Grewal, *loc. cit.*). Stout (*loc. cit.*) states that the difference between Europe and the Americas on the one hand, and the Asiatics on the other hand, may be partly accounted for by the fact that many internal cancers in the latter group are not diagnosed as such, thus raising the incidence figures of the external cancers; yet the discrepancy seems too great to be accounted for in this way. The explanation is probably to be found in the personal habits and cleanliness of the population concerned. In Ceylon the rarity of this lesion in Mohammedans, who are circumcized early in life, suggests the evil effects of the foreskin in the causation of this condition. Only 2 Mohammedans in our series presented this lesion, and in both there was a previous history of syphilis. White (*loc. cit.*) states that although penile cancer is the commonest carcinoma amongst Hindus it is almost unknown in the Mohammedans. Venereal diseases, especially if untreated, probably play a part in the ætiology as they help to produce irritants, which are retained behind a tight prepuce.

Although the largest number of cases occurred in the ten-year period 45 to 54 at least 19.4 per cent of the tumours were seen between the ages 35 and 44, and 8.5 per cent between 25 and 34 years. Three cases were seen between the age of 15 and 24 years. It would, therefore, appear that in Ceylon penile cancer is not restricted to elderly people.

7. *Prostate*.—Prostatic cancer accounted for 50 of the 317 primary carcinomata of the male generative organs. The largest number of cases occurred in the ten-year period 65 to 74, while the age-group 55 to 64 came a close second. No cases occurred below 44 years and the youngest was 47 years old. The average age at onset appears to be 60 to 70 years. The age incidence for carcinoma in our series was the same as for hypertrophy of the prostate.

All the sections examined were definitely carcinomatous—there being no border-line cases. The carcinomatous process was superimposed on a previous generalized fibro-adenomyoma of the gland, which is commonly called 'senile' hypertrophy of the prostate, and which is recognized to be associated with cancer. The series contained 146 glands of which 50 were found

to be carcinomatous, a percentage of 34·2 which is slightly higher than Swan's (1923) series, in which was found a malignancy rate of 25·7 per cent in 678 prostates and Barringer and Wildbolz's (1932) series where the malignancy rate was 20 per cent. Our figures, therefore, indicate the necessity of recognizing the enlarged prostate of the aged as a pre-cancerous condition. The removal of the gland on noticing signs of enlargement would not only afford a comfortable life to the aged, but also prevent the onset of cancer.

8. *Breast*.—Cancer of the mammary gland, although not prevalent to the same extent as in European countries, is of great importance on account of its easy accessibility and better opportunities afforded for its early discovery and removal, which is attended by less danger to life than in any of the internal cancers. There were 175 cases of mammary cancer in this series, 5 of which were in males.

Several workers (MacVicar, 1925, Bantu women of South Africa ; Lalung-Bonnaire and Bablet, 1925, Annamite women in Cochin (China) have reported that on account of different customs, habits of reproduction, lactation and the wearing of clothes, cancer of the female breast is rarer in the tropics than in Europe and America. In Ceylon too it appears to be rare, judging from admissions to the General Hospital, Colombo, to which many cases are referred to from all parts of the island, for operative treatment. Some idea regarding the comparative frequency of the condition can be obtained from Table XI which gives the admissions for mammary cancer to hospitals in other countries:—

TABLE XI.
*Incidence of mammary cancer according to hospital admissions
in different countries.*

Hospital.	Total cancer.	Mammary.	Percentage.
Presbyterian Hospital, New York ..	1,862	355	19
London County Hospital ..	15,068	2,495	16
General Hospital, Colombo ..	5,963	370	6

Age incidence.—The maximum number of cases occurred during the ten-year period 45 to 54 and more than half the cases between 35 and 54 years. In the very old (between 65 and 74) there were only 7 cases but in the young (between 25 and 34) there were 16 cases.

The histological classification of the tumours in this series is given in Table XII:—

TABLE XII.

Mammary carcinoma—histological classification.

Carcinoma simplex	151
Adenocarcinoma	18
Squamous carcinoma	1
TOTAL ..	170

Almost all the biopsy specimens were definite carcinoma, as in the case of the prostate, there being no border-line cases. Only two of these were considered to show early malignant changes. In only 15 was there evidence of chronic mastitis. The histological study of the remaining specimens did not throw any light regarding the origin of the growth. The prevalence of definite malignant changes in the biopsy material and the rarity of early malignant and pre-cancerous changes even in the case of an accessible site such as the breast, suggests the late arrival of cases to hospitals. This emphasizes the need for education on such lines as would result in early recognition being given to such a potential danger as the lump in the breast.

9. *Skin*.—Another primary site of importance not only on account of frequent involvement, but also because of its ready accessibility, was the skin. Cancer of the integument was noticed in the young as well as the old. It was seen twice as frequently in men as in women. Although definite ætiological factors in the development of skin cancer such as X-rays, heat, and chemical agents have been recognized in other countries, none of the cancers in our series could be attributed to these. In most of the cases the neglected chronic ulcer was the forerunner of carcinoma and in a few others the cancer took its origin from cysts and fistulæ or multiple warts especially in the perineal region, which became malignant either due to neglect or to the irritation caused by the remedies of the native physician. Quite a large proportion of growths were malignant melanomata which are more frequently seen in this country than in others. (Although malignant melanomata have been included in a separate group they will be discussed here as most of these arose primarily in the skin.)

Table XII-a gives the ætiological factor involved in the lesion.

TABLE XII-a.

Ætiological factors involved in the skin carcinomata.

		Males.	Females.	TOTAL.
Chronic ulcers	122	52	174
Perianal and anal warts	13	7	20
Sweat and sebaceous glands	6	..	6
Epidermoid cysts	3	1	4
Fistulæ in ano	5	..	5
Mastoid fistulæ	1	1	2
Umbilical fistulæ	1	..	1
Rodent ulcers	14	5	19
Baso-squamous carcinoma	10	5	15
Malignant melanoma	23	18	41
TOTALS	198	89	287

Of the skin cancers 61 per cent arose from chronic non-specific ulcers. This type of ulcer, especially of the leg, is a very common condition seen at all ages, in both sexes, all over the island. The ulcer usually arises as the result of trauma though not of very severe degree. It becomes infected on account of various local applications, and exposure to dust and dirt. Such ulcers grow larger and are found to be intractable to treatment. Malignant change supervenes on a large proportion of these. The danger of neglecting these ulcers, if made known to the public, will prove to be an important part in the prophylaxis of skin carcinoma.

An interesting neoplasm which is not uncommon in Ceylon is the basal cell carcinoma. Two histological types have been seen, one being the typical basal cell epithelioma, or rodent ulcer and the other the atypical form with 'pearl' formation, viz. the baso-squamous carcinoma. There were 19 of the former type and 15 of the latter. These were twice as frequently seen in men than in women, and were common in older people, the average incidence being about 60 years. The neoplasms were usually single but multiple rodent ulcers were seen in 2 cases. The common sites were the face, scalp, conjunctivæ and rarely the external auditory meatus and vulva. These are slowly growing tumours and the importance of recognition lies in the fact that they are amenable to radium and X-ray which have been employed in other countries with good results (Stout, *loc. cit.*).

The malignant melanoma of the skin occurs in this country more frequently than is realized. Although the pigmented mole that is frequently seen on the skin is the most liable

to form this type of growth, in Ceylon this does not appear to be the case, as the majority of these neoplasms arise from the sole of the foot, where innocent moles are not usually seen. However, in a few cases in women malignancy had supervened on a previously existing pigmented mole. Rapid enlargement of the mole darkening of its colour, ulceration and vascularity preceded the malignant changes. The situation of these tumours is given in Table XIII :—

TABLE XIII.

Malignant melanoma—anatomical distribution.

			Males.	Females.	TOTAL.
Foot (plantar surface)	22	8	30	
Leg	1	1	
Thumb	1	1	
Abdominal wall	1	1	
Neck	1	..	1	
Face	1	1	
Ear	1	1	
Vulva	1	1	
External urinary meatus	1	1	
Gum	1	1	
Eye	1	1	
Unknown site	1	1	
			<hr/>	<hr/>	<hr/>
TOTALS	23	18	41	

In males all the malignant melanomata, excepting one, arose on the plantar surface of the foot either the heel or the sole or the plantar aspect of the toes. In females more than half the growths arose from the same site, the remaining tumours arising from previously existing innocent moles. (The tumour of the eye probably arose from the choroid, which is a favourite site of origin of these tumours.) The plantar surface of the foot which is frequently subjected to trauma in the case of bare-footed individuals was the favourite site of origin of this tumour. All the tumours in this situation, excepting three, were found in people who walked bare-footed and whose feet were thus continually exposed to injury. Almost in every case the pigmented ulcer followed some injury to the foot either by a piece of stone or wood, or a prick by a thorn. The native physicians treat these by various local applications. The ulcers become larger and when the patient is seen in the hospital, a large melanotic growth with multiple secondaries is noticed. The site of the lesion on a part which is very liable to be injured suggests trauma as a possible ætiological factor in those malignant moles, which do not arise from innocent moles. The local applications of the native physicians perhaps act as irritants.

Melanoma malignum especially of the foot appears to be a condition which is somewhat peculiar to Ceylon. It is rather a curious fact that several authorities (Stout, *loc. cit.*; Mackee and Cipollaro, 1937; Pack, 1939) have stated that this tumour is exceedingly rare in the negro. The quantity of pigment in any particular race has therefore no bearing on the tumour. As excision and radiotherapy are useless in this condition the most important treatment is prophylaxis. As a general rule, any mole which is of such a size and consistency that it may be irritated, should be removed and as trauma appears to be a causative factor in melanoma of the foot, due attention should be paid in our hospitals to traumatic ulcers especially of the plantar aspect.

10. *Thyroid*.—Cancer of the thyroid accounted for 2·1 per cent of the malignant tumours examined. Tumours which have been looked upon as simple adenomata have often been

found histologically to be malignant. The biopsy material examined from the thyroid gland consisted of the following (Table XIV) :—

TABLE XIV.
Tumours of thyroid gland.

<i>Benign tumours—</i>					
Colloid goitres	98*
Cystadenomata	13
Fœtal adenomata	28
Toxic goitre	39
Lymphadenoid goitre	6
					184
Carcinomata	38
TOTAL					222

* It is not known how many of these were toxic as most of the cases had received iodine treatment prior to operation.

Of the biopsies from the thyroid gland 17 per cent were found to be malignant. The evidence of cancer in biopsy material is very much higher than de Courcy's (1924) series in which only 1·2 to 2 per cent of all operated goitre cases were malignant. As cancer usually develops in a pre-existing goitre, the high incidence of cancer in glands removed surgically indicates that the hyperplastic process had continued at least for some time before any intervention. Just as hyperplasia is commoner in women so, too, is cancer. In this series there were 24 females and 14 males, a proportion of nearly 2 : 1.

The common age for cancer according to our figures is between 45 and 64. However, 2 cases (male and female) occurred before 20 years. The histological types seen in this series were the adenocarcinoma, the malignant papillary cystadenoma and the solid alveolar carcinoma. In one case the cancer arose from an aberrant thyroid which is said to be very rare (Stout, *loc. cit.*). Metastasis from thyroid cancer were found in the skull, ribs, lung, the cervical and axillary glands.

One of the main principles in the treatment would be the recognition of all adenomata as potential cancers and the surgical removal of these. By so doing it is probable that some individuals will not develop cancer, who would have done so if the adenoma had not been removed.

LYMPHOID TISSUE.

Malignant disease of lymphoid tissue accounted for 55 tumours and was composed of the following varieties: Lymphadenoma 32; lymphosarcoma 18; lympho-epithelioma 5.

(a) *Lymphadenoma*.—This condition appears to be more common in Ceylon than our figures would indicate, as a biopsy is done only to establish a diagnosis. There were 26 males and only 6 females in this series. The glands are affected at a young age, the largest number of cases in both sexes, viz. 14, occurred in the age period 15 to 34. Four cases in males occurred between 5 and 14 years and one in a male aged 4 years. Our findings regarding age and sex incidence are the same as recorded by Minot and Isaacs (1926) in a study of 173 cases of Hodgkin's disease. Amongst the females, the Tamils showed a greater tendency to be effected, there being only 2 Sinhalese to 4 Tamils.

(b) *Lymphosarcoma*.—There were 18 cases of lymphosarcoma 14 being in males and 4 in females. This too appears to affect younger people, as 8 of these were noticed in patients under 20 years of age. In addition to the lymphatic glands, the tonsil, liver, kidneys, pericardium, intestines, retro-peritoneal and retro-orbital tissues were involved in the process.

(c) *Lympho-epithelioma*.—A less well-defined but interesting category of tumours was the lympho-epithelioma. There were only 5 such tumours in this series and 3 took origin from the tonsil and 2 from the nasopharynx, situations where the covering epithelium is in very intimate relationship with lymphoid tissue. Our collection is too small to draw any conclusions.

THE SARCOMATA.

Tumours of the connective tissue, the sarcomata, were not so frequently seen as those of epithelial origin. There were only 188 or 8.2 per cent of the total malignant tumours (*vide* Table III).

In a complex group of tumours like the sarcomata, it is not possible, especially in the late stages of the growth, to be exact regarding the tissue from which it arose, on account of the marked degree of de-differentiation. It seems probable that of the 188 sarcomata only 20 were primarily in bone, the remaining 168 being in the soft tissues. Of the former group the largest number were to be found in long bones. In the latter group 35 were found arising from muscles and fasciæ of the lower limbs. Fifteen were neural in origin and 11 arose from the female breast. The retro-peritoneal tissue of the abdomen gave origin to 10 tumours.

Table XV gives a morphological classification of the sarcomata in this series:—

TABLE XV.

Sarcomata.

Histological classification, sex and racial distribution.

		MALES.					FEMALES.					Total.
		Sinhalese.	Tamils.	Burghers.	Moors.	Malays.	Sinhalese.	Tamils.	Burghers.	Moors.	Malays.	
Round cell sarcoma	..	5	1	1	7
Spindle cell	..	12	4	..	1	..	5	1	23
Mixed cell	1	1
Polymorphous	..	10	3	3	1	17
Fibrosarcoma	..	18	6	1	9	2	1	37
Chondrosarcoma	..	5	1	4	10
Myxosarcoma	..	10	2	5	2	2	21
Osteosarcoma	..	2	2
Angiosarcoma	..	5	2	7
Myosarcoma	..	2	1	3
Ewing's sarcoma	..	2	2	4
Unclassified	21	5	1	2	..	24	..	2	1	..	56
TOTALS		92	23	2	3	1	55	6	5	1	..	188

Sex and age incidence.—Males were affected nearly twice as frequently as females and these neoplasms were met with at all ages. The largest number in both sexes occurred between 25 and 44 years, 5 cases were seen under 5 years of age, 3 of these were in infants aged two, six and seven months and 2 in children aged three years and three years nine months. Thus, children and young adults were found to be more frequently afflicted than in the case of carcinoma.

SPECIAL TUMOURS.

On account of their rarity only 22 malignant neoplasms have been classified as special tumours (*vide* Table XVI).

TABLE XVI.
Special tumours.

	MALES.					FEMALES.					Total.
	Sinhalese.	Tamils.	Burghers.	Moors.	Malays.	Sinhalese.	Tamils.	Burghers.	Moors.	Malays.	
Retinoblastoma ..	3	1	..	1	..	4	1	..	1	..	11
Neuroblastoma ..	3	1	4
Spongioblastoma multiforme ..	1	1	2
Adenocarcinoma (bronchus) ..	1	1
Teratoblastoma (kidney) ..	1	1
Plasmacytoma ..	1	1
Malignant osteoclastoma	1	1
Undifferentiated carcinoma	1	1
TOTALS ..	10	2	1	1	..	6	1	..	1	..	22

The most prevalent malignant tumour in this group was the retinoblastoma. There were 11 such cases, 5 being in males and 6 in females. It occurred only in the Sinhalese, Tamils and the Moors. All the cases seen were in children under 5 years of age. Although it is stated that these tumours are hereditary, no such history was obtained from these cases.

Although malignant disease is looked upon as afflicting middle-aged and elderly persons it is not fully realized that comparatively young people may be its unfortunate victims. In all countries a certain proportion of young people and children suffer from malignant growths. Tumours, such as the retinoblastoma, the neuroblastoma, hæmangio-endothelioma and Ewing's sarcoma, are known to occur in young people.

Our analysis shows that 120 out of 2,295 malignant growths or 5·2 per cent of the tumours occurred in persons under 20 years of age. There were 66 males and 54 females in this group.

The phenomena connected with growth predominate in this period and neoplasms, which take a hold at a time of acceleration of the general growth processes, grow much more rapidly than at other periods of life and metastasize more easily. The tumours occurring during this period have been studied in respect to their incidence during infancy, childhood, pre-sexual and sexual periods.

Table XVII gives the histological types of the neoplasms occurring at these periods and Table XVIII gives the anatomical site:—

TABLE XVII.

Histological classification of the malignant tumours occurring at various periods in persons under 20 years.

Infancy (from birth to 10 months), 8 tumours—

Hæmangio-endothelioma	4
Angiosarcoma	1
Teratoblastoma (kidney)	2
Sarcoma	1
TOTAL	8

TABLE XVII—*concl'd.**Childhood (11 months to 8 years), 33 tumours—*

Retinoblastoma	11
Sarcoma	5
Carcinoma	4
Lymphadenoma	3
Neuroblastoma	3
Malignant endothelioma	2
Lymphosarcoma	2
Malignant melanoma	2
Teratoblastoma (kidney)	1
TOTAL					33

Pre-sexual (9 years to 15 years), 29 tumours—

Sarcoma	12
Carcinoma	7
Lymphadenoma	2
Ovary: Teratoma 1	4
Dysgerminoma 1	
Arrhenoblastoma 1	
Granulosa cell tumour 1	
Lymphosarcoma	1
Lympho-epithelioma	1
Hæmangio-endothelioma	1
Brain (glioblastoma)	1
TOTAL					29

Sexual (16 years to 20 years), 50 tumours—

Carcinoma	27
Sarcoma	10
Lymphadenoma	2
Endothelioma	2
Ovary (dysgerminoma)	2
Testis: Seminoma 1	2
Teratoma 1	
Neuroblastoma	2
Chorion epithelioma	2
Malignant melanoma	1
TOTAL					50

TABLE XVIII.

Anatomical distribution of malignant tumours in persons under 20 years.

Eye	17
Soft tissue (sarcoma)	16
Female genital organs—ovary 10; uterus 3; fallopian tubes 2; vulva 1	16
Lymphoid tissue	15
Skin—carcinoma 9; malignant melanoma 2	11
Male genito-urinary tract—testis 3; adrenals 3; kidney 3	9
Blood vessels	9
Bones (sarcoma and Ewing's tumour)	7
Buccal cavity	7
Salivary gland (parotid)	4
Breast	2
Thyroid	2
Fistulæ—umbilical 1; mastoid 1	2
Liver	1
Brain	1
Tonsil (lympho-epithelioma)	1
TOTAL					120

It will be seen from Tables XVII and XVIII that a particular histological type was prevalent at each period; thus, the most frequent neoplasms observed during infancy were the blood-vessel tumours in childhood, the retinoblastoma, in the pre-sexual period, the sarcoma, and in the sexual period, the carcinoma. Of carcinoma 38 cases were noticed during this period of life—a significant fact on account of the comparative rarity of this type below the age of 20.

Regionally the eye appeared to be the organ most frequently involved by malignant disease in the young.

SUMMARY AND CONCLUSIONS.

1. Although the incidence of malignant disease cannot be accurately estimated it appears to be sufficiently common in Ceylon to warrant further study.

2. Out of 10,880 consecutive biopsies 2,295 or 21.3 per cent showed evidence of malignant disease. The incidence of malignant tumours in biopsy material does not differ much from that recorded in India.

3. The primary carcinomata constituted 79.1 per cent of the malignant tumours and arose in 31 different sites. A high incidence of carcinoma in biopsy material was noticed in the following organs: The generative organs in both sexes (44.2 per cent), the cervix and penis alone (17.4 and 13.6 per cent respectively), the buccal cavity (15.1 per cent), skin (13.6 per cent) and the breast (9.6 per cent). The gastro-intestinal tract including the liver contributed only 3.4 per cent.

4. The predominating histological type was the squamous-cell carcinoma.

5. Although, as in other countries, females were seen to be more frequently affected, there was not much disparity in the sex incidence on account of the high incidence of cancer of the penis.

6. Our figures regarding racial incidence indicated only that people who were better informed of cancer sought hospital treatment at an early stage of the disease and did not wait till the carcinoma was inoperable or so obvious that a biopsy was rendered unnecessary.

7. The maximum incidence of carcinoma in both sexes appeared to be in the ten-year age-groups from 45 to 54, which is earlier than occurs in Japan, most European countries and the United States of America. The explanation for this is probably to be found in the fact that the expectation of life in the population of Ceylon is lower than that of the other countries.

8. Carcinomata occurring at certain primary sites have been discussed more fully. Some of the unusual features noticed were:—

(a) A very high incidence of carcinoma of the cervix and penis in biopsy material.

(b) The greater prevalence of certain types of tumours, such as the chorion epithelioma and the malignant melanoma, in Ceylon as compared with other countries.

(c) The greater frequency of malignant changes found in ovarian cysts, thyroid and prostate glands.

(d) The tendency for ovarian cancer to occur in women between 25 and 34 years.

(e) The tendency for a large proportion of skin cancers to arise from chronic ulcers.

9. Malignant tumours of connective tissues (the sarcomata), lymphoid tissue and a group which, on account of its rarity, has been called 'special tumours' have been discussed. Sarcomata occurred in younger age-groups than the carcinomata and its incidence was found to correspond to that of Western countries and was much lower than that in India. Tumours of the lymphoid tissue also occurred in the young. The retinoblastoma was the most prevalent amongst the rare malignant tumours.

10. Malignant tumours in those under 20 years of age, which formed 5.2 per cent, have been discussed in respect to their incidence during infancy, childhood, pre-sexual and sexual periods. The more prevalent types characteristic of each of these periods have been indicated. The eye appeared to be more frequently involved than the other organs in this group.

11. Probable factors governing the causation of cancer in certain situations have been suggested. Our study has indicated that the controllable carcinogenic factor, viz. continued irritation, operated on particular sites such as the cervix, oral cavity, penis and skin. This consideration brings a large proportion of cancers in Ceylon (59.7 per cent of the carcinomata in our series) under the group of preventable diseases.

12. The control of cancer in Ceylon does not appear to be so complex as in other countries, as a large proportion of the carcinomata (59.7 per cent in our series) arose in sites, which, on account of easy accessibility, were amenable to either surgical treatment or radium therapy. The difficulty, however, was the late arrival of cases to hospitals as shown by this study. Only 161 of the 2,295 malignant tumours (7 per cent) could be regarded as early malignancy or pre-cancer.

The results that could be achieved by any form of therapeutic procedure is therefore necessarily limited. The general public should be enlightened regarding the intimate nature of the signs and symptoms which may mean cancer. The potential danger of neglecting a vaginal discharge, a sore on the penis, a wound or ulcer on the leg, a carious tooth or a polypus, and the relationship that these conditions bear to malignant disease should form a part of the educational propaganda in a campaign against cancer.

13. Whether a large proportion of sufferers from malignant disease are first seen by ayurvedic practitioners is a question which deserves investigation. While conducting an inquiry of this nature we must not fail to perfect our own organizations. Instruction in the early diagnosis of cancer should be an important feature in our medical curriculum and the necessary equipment for such diagnosis should be made available to every medical man.

Our hospital case records should be complete and should in every case include the reports of histological examinations. As the efficacy of any therapeutic procedure for malignant disease could only be judged by periodic examination of the patient, there should be a follow-up system whereby a cancer patient may be observed for at least 5 years after treatment.

These measures involve a great deal of perseverance, energy, patience and money. The sacrifice, however, will not be too great if we can be satisfied that a real gain has been made against a disease which has so stubbornly resisted the continued attack of sanitary science, bacteriology, chemistry, physics and even surgery.

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BLOOD AND BONE-MARROW IN PRE-CIRRHOTIC TOXIC SPLENOMEGALY DUE TO MANGANESE.

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FINDLAY (1924) was the first to use manganese chloride to get a precise knowledge of the toxic effects of manganese on animals. Experimenting on rabbits, he noticed enlargement of the spleen. Handovsky *et al.* (1925-26), Hurst and Hurst (1928), Rao (1931), Albot (1931), Vandre Schueren (1932) are the other workers as quoted by Moon (1934) who used the same drug. They injected the chemical for inducing cirrhosis of the liver and did not make any mention about the splenic changes. Menon (1938) used this drug to study the changes in the spleen. He found fibrotic and proliferative changes in the spleen and definite splenomegaly independent of portal stasis and hepatic cirrhosis. He opined that the changes in the spleen resembled those in splenic anæmia. Cameron and de Saram (1939) also noticed splenomegaly with pulp hyperplasia in addition to hepatic damage, after administering carbon tetrachloride to rats with marsupialized spleens having no portal connection. Changes in the spleen, comparable to those seen in Banti's disease, in the absence of progressive anæmia and leucopenia do not help us to understand the pathology of splenic anæmia. So far, none of the workers has made any reference to blood and bone-marrow changes. The present investigation has been particularly directed to the study of these changes.

Method and material.—Thirty-five young rabbits of weights varying from 0.8 kg. to 1.6 kg. were used for the investigation. Of these nine were kept as control animals. The animals were lodged in an airy room in separate cages and given uniform diet. Chemically pure manganese chloride dissolved in normal saline in strengths of 0.5 per cent and 1 per cent solution was injected intramuscularly at specified intervals. The animals were divided into two batches. The first group was injected with 15 mg. to 30 mg. of manganese chloride per kilo body-weight every third day, whilst the second group received 8 mg. to 12 mg. on alternate days. A single dose of 30 mg. per kilo body-weight proved fatal in one animal, and two others died after two injections of 25 mg. each. These animals developed severe diarrhœa before death. A few other animals died while receiving the injections due to various causes such as peritonitis, chronic diarrhœa and abscess formation at the sites of injection. Findlay (*loc. cit.*) noticed a fall in weight in all his experimental rabbits, with manganese intoxication. Though there was an initial fall in weight in our animals, a large number of them gained weight with time. Much stress was laid on the high incidence of coccidiosis in rabbits by Moon (*loc. cit.*); but in our animals only one showed this infection. The animals which survived the injections were killed by cervical dislocation. The tissues were fixed in 10 per cent formol saline and the sections were stained by (1) Ehrlich's hæmatoxylin and eosin, (2) Weigert's iron hæmatoxylin and van Gieson, (3) Heidenhain's azan stain, (4) Foot and Menard's method of silver impregnation of reticulum tissue, (5) Sudan III, (6) Perl's prussian blue stain counterstained with van Gieson, and (7) Leishman's stain for tissues.

Blood.—Before starting the animals on manganese chloride injections, a preliminary comprehensive hæmatological examination consisting of an erythrocyte count, hæmoglobin estimation, total and differential leucocyte counts, platelet count and reticulocyte percentage was done. These examinations were repeated for all the animals including the controls, in the

beginning twice weekly and later, once in a fortnight. Table I shows the averages of the hæmatological readings for our normal rabbits. Our blood normals agree with those of Sabin *et al.* (1936) and Craigie (quoted by Kolmer and Boerner, 1938). The lymphocytes in the rabbit's blood are as a rule more numerous than in the human blood. It is difficult to differentiate between a monocyte and a lymphocyte in a rabbit, as the former cell fails to show any granules unless stained supravital. Our monocyte counts are less than those of Sabin *et al.* (*loc. cit.*) probably since supravital staining was not used.

TABLE I.
Average blood counts of 9 normal rabbits.

R.b.c., per c.mm.	W.b.c., per c.mm.	Platelets, per c.mm.	Reticulocyte, per cent.	Hæmoglobin, per cent.	Hæmoglobin, g.	Polymorphs, per cent.	Lymphocytes, per cent.	Basophils, per cent.	Eosinophils, per cent.	Monocytes, per cent.
5.18 mils.	6,100	276,000	1.2	76	12.10	41	58	0.50	0.3	0.3

The full record of hæmatological counts performed during the period of manganese intoxication is omitted to economize space. None of the experimental animals developed any appreciable anæmia at any stage. In rabbit No. 28, the red blood cell count shot up from 5.58 million to 7.51 million per c.mm., the hæmoglobin content increased from 85 per cent to 98 per cent and the reticulocytes from 0.6 per cent to 2.4 per cent. In the rest of the animals, the variation in the red blood cell counts was within one million and the hæmoglobin percentage within 10. Immature forms of erythrocytes were never met with in the peripheral blood, nor was there an increase in the reticulocytes to indicate rapid regeneration of red blood cells. A moderate degree of leucocytosis was seen in a large number of animals. Inflammatory reaction of the tissues at the site of injections may be responsible for this.

Bone-marrow.—Our study of bone-marrow had to be restricted to an examination of the material obtained after the death of the animals, due to difficulty in getting the marrow substance during life. Both macroscopic and microscopic studies of the bone-marrow in selected bones as femur, tibia, sternum and ribs were made. In the long bones of a normal rabbit the red marrow is more marked towards the ends, progressively diminishing towards the centre, where it is fatty. No appreciable macroscopic changes in the bone-marrow were noticed in twenty of the twenty-six injected animals. Of the six that showed changes, in rabbits Nos. 1, 12 and 20, there was slight increase in the fatty tissue and their peripheral blood showed a slight fall in the r.b.c. and hæmoglobin content. In rabbits Nos. 4, 15 and 16, the red marrow appeared increased, but there were no changes in the blood counts.

Sections of the bone-marrow stained with hæmatoxylin-eosin and Giemsa stain provided material from which to estimate the total amount of hæmopoietic tissue. Custer's method of staining was not quite successful in our hands. As identification of the individual cells in sections was not always easy, stained smears were utilized for differential counts. In almost all cases, these results were in agreement with those observed in the stained sections. Animals that received repeated large doses of the chemical, showed generalized congestion of the marrow and degenerative changes of a few fixed mesenchymal cells which form the reticular stroma. The nuclei of these cells showed karyorrhexis and pyknosis. Some of the megakaryocytes also showed nuclear condensation (Plate II, figs. 1, 2). Scattered all over, were found large phagocytic cells containing hæmosiderin pigment. The bone-marrow of animals that received

PLATE II.

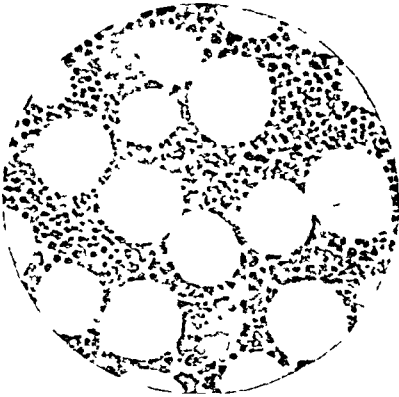


Fig. 1—Oil immersion photomicrograph of normal bone marrow stained with hematoxylin and eosin

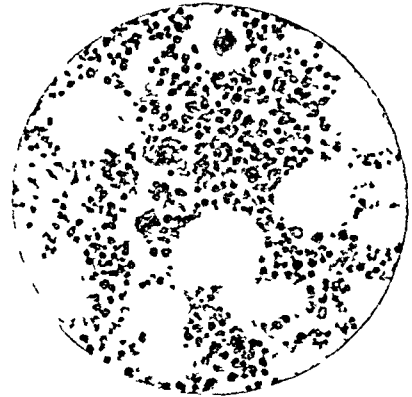


Fig. 2 Oil-immersion photomicrograph of the bone marrow of a rabbit which has received large toxic doses of manganese chloride. Nuclear condensation of megakaryocytes and mesenchymal cells is seen.

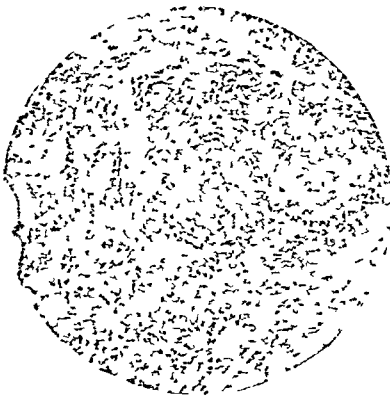


Fig. 3—Low power photomicrograph of the spleen of a normal rabbit.

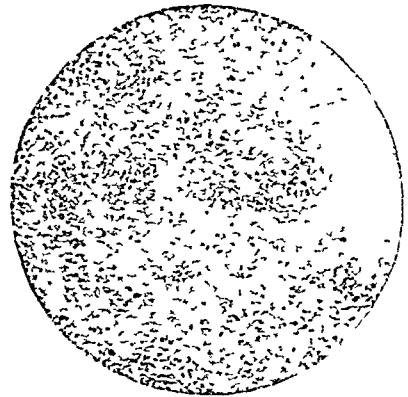


Fig. 4.—Low power photomicrograph of the spleen of a rabbit which has received repeated large toxic doses



Fig. 5—Medium power photomicrograph of the same section as in fig. 4 to show the extent of damage in the Malpighian bodies and the splenic pulp

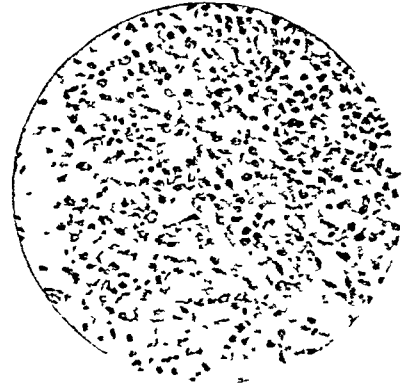


Fig. 6—Oil-immersion photomicrograph of the splenic pulp of a rabbit which has received repeated large toxic doses. Shows pulp cell damage and obliteration of sinusoidal spaces due to swollen Billroth cords

small doses did not show any noteworthy change. In a few cases, the marrow appeared to be more cellular, suggesting a hyperplastic reaction but the differential counts were unchanged. Even in the case of rabbit No. 28 which had received 966 mg. of manganese chloride and in which the peripheral blood showed increase in the erythrocytes, the bone-marrow appeared perfectly normal. The quantity and distribution of fatty marrow appeared unaltered. Sections treated with Foot and Menard's method of silver impregnation and Heidenhain's stain failed to show any gross increase in the reticulum or connective tissue fibres. In rabbit No. 4, which received 1,010 mg. of manganese chloride, an increased amount of connective tissue was found both in the walls of the blood vessels and sinusoids, but much importance cannot be attached to the above findings as other animals that received equally heavy doses failed to show similar changes. Hæmosiderin pigment in bone-marrow sections was seen well when stained with Perl's prussian blue and counterstained with van Gieson. Even the normal bone-marrow contained a fairly large amount of this pigment and there was no appreciable change in the bone-marrow of animals subjected to manganese intoxication.

Differential counts of the cells of the bone-marrow.—As stated by Sabin and her co-workers (*loc. cit.*) the bone-marrow cell counts cannot be as accurate as blood counts, since the cells are not always spread evenly and only select fields can be utilized. A minimum of 500 cells were counted in each case. We followed the classification of Young and Osgood (1935) recommended by Whitby and Britton (1937) with a slight modification. We did not count erythroblasts separately as done by Young and Osgood, but included them with megaloblasts. Most of the rabbits used for our experimental work were above two months in age, and the average of the counts of our control animals agreed in essentials with those of Sabin and her co-workers. An examination of Table II shows that the differential counts of the bone-marrow in injected animals did not in any way differ from the normals in control animals, apart from some minor variations which are bound to occur. The findings in four animals, viz. 10, 18 in group I, and 19, 35 in group II, are omitted as early post-mortem decomposition prevented a reliable study. Our lymphocyte counts are slightly higher than those given by Sabin and her co-workers (*loc. cit.*). The normal ratio between the erythroid and the myeloid series of cells was unaltered.

Study of spleen.—After the removal of the spleen, it was weighed and the size measured. The weight of normal spleens in control animals varied between 0.3 g. and 0.4 g. per kilo body-weight, except in rabbit No. 13, in which it was 0.63 g. The average weight for our normal animals worked out at 0.35 g. per kilo body-weight, which is less than that of Krumbhaar.

Group I : Repeated large doses.—Eighteen rabbits were given large doses (15 mg. to 30 mg. per kilo body-weight) of manganese chloride of 1 per cent strength. Of these, one animal (rabbit No. 18) was found at post-mortem, infected with coccidiosis. In the majority of animals, there was a moderate increase in the weight of the spleen, but it was not uniform. The capsule and trabeculæ showed an increased amount of connective tissue. The Malpighian follicles were atrophic with irregular margins. The germ centres were absent and the lymphocytes of the follicles were compact, with deep staining nuclei. Nuclear changes like karyorrhexis and pyknosis were noticed in some of the cells, chiefly in the peripheral zone of the Malpighian follicles. In some cases all that was left behind, was the central artery with a few scattered lymphocytes in its neighbourhood (Plate II, figs. 3, 4, 5). Often, the endothelium of the vessels was swollen with narrowing of the lumen. In markedly congested spleens (rabbits Nos. 15, 16, 27, 28, 29 and 33) the peripheral zone of the Malpighian follicle showed erythrocytic infiltration. Early fibroblastic reaction was noticed near the marginal zone of the Malpighian follicles. This was found to extend along the branches of the central artery, some of which curve round the periphery of the follicles, and the terminal penicillar arteries that ultimately merge into the splenic pulp. Degenerative changes were also seen in the pulp syncytium. The Billroth cords were swollen, encroaching on the sinusoidal spaces. The swollen pulp cells showed either granular degeneration or coagulative necrosis of their cytoplasm (Plate II, fig. 6). Some of them contained large droplets of fat. The nuclei of a few of these cells showed degenerative changes similar to those seen in the marginal zone of the Malpighian follicles. These changes

TABLE II.

Bone-marrow : mean values of differential counts in each group.

Site of bone-marrow.	Megaloblasts, per cent.	Normoblasts, per cent.	Myeloblasts, per cent.	Premyelocytes, per cent.	Myelocytes, per cent.	Metamyelocytes, per cent.	Polymorphs, per cent.	Basophilic myelocytes, per cent.	Basophilic meta-myelocytes, per cent.	Basophils, per cent.	Eosinophil myelocytes, per cent.	Eosinophils, per cent.	Lymphocytes, per cent.	Monocytes, per cent.	Megakaryocytes, per cent.
Average normal counts.	0.4	48.6	0.3	3.0	19.0	8.1	14.4	0.6	0.2	0.2	0.3	0.2	12.1	0.5	0.3
GROUP I: Rabbits Nos. 8, 9, 11, 12, 14, 15, 16, 22, 23, 24, 25, 27, 28, 29, 31 and 32. Repeated large doses, 15 mg. to 30 mg. per kilo body-weight (1.0 per cent solution).															
Femur ..	0.4	43.0	0.25	3.5	18.4	8.4	8.6	0.40	0.2	0.1	0.2	0.1	14.6	0.6	0.4
Sternum ..	0.5	44.0	0.32	3.8	17.5	8.4	8.5	0.35	0.2	0.1	0.3	0.1	14.5	0.7	0.4
GROUP II: Rabbits Nos. 1, 3, 4, 20, 33 and 36. Repeated small doses, 10 mg. to 15 mg. per kilo body-weight (0.5 per cent solution).															
Femur ..	0.4	49.0	0.25	3.3	13.0	8.5	13.2	0.6	0.1	0.1	0.4	0.1	10.6	0.5	0.6
Sternum ..	0.5	47.5	0.40	3.8	12.4	8.1	14.3	0.5	0.1	0.2	0.4	0.1	11.2	0.5	0.6

were not limited to the vicinity of the Malpighian follicles, but spread diffusely into the pulp syncytium. The reticulum cells lining the sinusoids were intact, with a few of them protruding prominently into the lumen, due to their swollen condition. Large macrophage cells containing hæmosiderin pigment and occasionally entire erythrocytes were seen both in the pulp mesh and the sinusoidal spaces. Hæmorrhages were rare. Siderotic nodules were never met with. Rabbit No. 14 which died after a single injection of 30 mg. showed similar changes. There was no fibroblastic reaction, but it is easy to understand, as there was no time for recovery. As rabbit No. 24 developed severe diarrhœa after 3 injections, further injections were suspended and the animal was allowed to recover. Later, when the animal was killed, the spleen presented a normal appearance, but for a moderate increase in fibrous tissue indicative of preliminary damage. Rabbits Nos. 12, 28 and 29 failed to show the changes associated with this group. In rabbit No. 12, the appearance of the spleen was normal, while in rabbits Nos. 28 and 29, the splenic tissue showed hyperplastic changes similar to those seen in the later stages of group II.

In a few experimental animals an increase of argentophilic fibres was found in the Malpighian follicles and sinusoidal walls, while in others there was no appreciable change.

Perl's prussian blue stain counterstained with van Gieson helped to identify and localize the hæmosiderin pigment. A large amount of this pigment is always found in the spleen of a normal rabbit. There was no evidence of increase in the hæmosiderin content in the spleens of experimental animals.

Group II: Repeated small doses.—Eight rabbits received 8 mg. to 12 mg. each of manganese chloride per kilo body-weight for a period of 18 to 283 days. Only in one case the spleen had enlarged to one and a half times the normal size and weight. In the rest, there was no appreciable change even though one of them received on the whole 1,010 mg. in 283 days (Table III).

Microscopic changes in the spleens of rabbits which died early were of degenerative nature, similar to those seen in group I. In the rest, that survived longer, the splenic capsule was thickened and the trabeculæ prominent. The trabecular vessels and the central arteries of the Malpighian follicles showed moderate intimal thickening with narrowing of the lumen. There was hyperplasia of the Malpighian follicles with slight increase of connective tissue in the vicinity of the marginal zone.

From this, we are to conclude that the early degeneration of splenic parenchyma is followed by proliferation and regeneration. The spleen evidently gets used to the toxin when administered in small doses and the damage done in the early stages is repaired.

Study of the liver.—We have studied in detail the changes in the liver of the different batches of animals. Our main findings are in conformity with those recorded by previous workers. Briefly, the changes are (1) animals that died after a single large dose of the drug showed extensive fatty degeneration and necrosis of the liver cells; (2) animals that were injected with large toxic doses and killed at intervals, showed both degenerative and proliferative changes. There was fatty degeneration of the liver cells at the periphery of the lobule extending from $\frac{1}{3}$ rd to $\frac{2}{3}$ rds its thickness. In a few cases it was more extensive and not localized to any particular zone. Necrotic changes were also in evidence. In animals that were killed after several injections, there was unmistakable evidence of regeneration of the parenchyma cells. Most of the lobules were surrounded by an abundant newly formed tissue, rich in collagen fibres and innumerable proliferating bile capillaries. The biliary epithelium of some of these vessels was thrown into folds. In places, the newly formed fibrous tissue was found to penetrate the liver lobules. The portal vein radicles were dilated. The entire picture was suggestive of monolobular cirrhosis of biliary type. Rabbits Nos. 12, 28 and 29, belonging to this group, failed to show any change and it is interesting to note the same animals were negative for any splenic changes.

Group II: Repeated small doses.—Animals killed after a few injections showed changes in the liver similar to those described in group I, but in those that survived long, the liver was free from frank cirrhotic changes. Evidently the animal gets used to the drug, when given in small doses and the early damage is repaired beyond recognition.

TABLE III.

Serial number.	Rabbit number.	Weight of the rabbit, kg.	Amount of manganese chloride for each injection, mg./kg.	Total amount of manganese chloride given, mg.	Time of death after the first injection.	Size of spleen, cm.	Weight of spleen, g.	Estimated normal weight of spleen, g.	Mean enlargement.
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GROUP I: *Repeated large doses, 15 mg. to 30 mg. per kilo body-weight (1.0 per cent solution).*

1	8	1.30	20	182	18 days	4.0 × 0.8 × 0.4	0.50	0.45	1.1
2	9	1.60	20	466	52 "	6.0 × 1.2 × 0.5	2.00	0.56	3.6
3	10	1.60	25	240	20 "	4.3 × 0.7 × 0.3	0.65	0.56	1.15
4	11	1.25	15	905	151 "	4.3 × 0.8 × 0.5	0.80	0.44	1.8
5	12	1.40	15	568	84 "	4.5 × 0.8 × 0.4	0.55	0.40	1.1
6	14	0.75	30	23	30 hours	3.8 × 0.6 × 0.3	0.39	0.26	1.5
7	15	1.50	20	674	79 days	3.5 × 0.9 × 0.4	0.50	0.52	1.0
8	16	1.25	20	270	38 "	5.8 × 1.0 × 0.7	1.05	0.44	2.4
9	18	1.70	25	86	4 "	4.5 × 0.8 × 0.3	0.90	0.60	1.5
10	22	1.50	15	592	87 "	5.2 × 1.1 × 0.7	1.40	0.52	2.7
11	23	1.40	15	595	87 "	4.0 × 0.8 × 0.5	0.60	0.49	1.2
12	24	1.45	25	112	18 "	3.9 × 0.9 × 0.45	0.40	0.51	..
13	25	1.60	25	80	6 "	3.5 × 0.7 × 0.3	0.45	0.56	..
14	27	1.25	20	102	10 "	4.5 × 1.1 × 0.3	1.00	0.44	2.3
15	28	1.45	15	966	162 "	3.5 × 0.8 × 0.35	0.62	0.51	1.2
16	29	1.35	15	964	163 "	3.7 × 0.8 × 0.4	0.45	0.47	1.0
17	31	1.20	20	380	56 "	3.3 × 0.7 × 0.4	0.35	0.42	..
18	32	1.35	20	670	93 "	4.7 × 1.15 × 0.4	0.82	0.47	1.8

GROUP II: *Repeated small doses, 10 mg. to 15 mg. per kilo body-weight (0.5 per cent solution).*

1	1	1.15	10	255	53 days	3.0 × 0.5 × 0.3	0.30	0.40	..
2	3	1.10	10	255	102 "	3.5 × 0.9 × 0.3	0.37	0.38	1.0
3	4	1.65	10	1010	283 "	4.0 × 0.8 × 0.4	0.65	0.58	1.1
4	19	1.50	8	840	180 "	4.2 × 0.6 × 0.3	0.25	0.52	..
5	20	1.20	8	438	103 "	3.5 × 0.7 × 0.3	0.45	0.42	1.0
6	33	1.65	12	420	52 "	4.5 × 0.85 × 0.35	0.65	0.58	1.1
7	35	1.30	10	180	26 "	4.3 × 0.8 × 0.35	0.65	0.45	1.4
8	36	1.45	10	134	18 "	4.2 × 0.8 × 0.4	0.72	0.51	1.2

DISCUSSION.

A detailed study of the degenerative changes in the liver and spleen of rabbits after administering manganese chloride has been made by various workers. The chief finding was initial toxic necrosis, followed by regenerative and fibrotic changes. The splenic changes were

coincident with the liver damage, the toxin being brought simultaneously to both the organs through the blood stream. Splenomegaly with fibrosis, observed in animals subjected to prolonged intoxication, has resemblance to human spleen in Banti's disease (Menon, *loc. cit.*). Similar changes are reported to be present in Bengal splenomegaly described by De (1932); but this condition is almost always associated with an irregular fever. We have made, in addition, a thorough study of blood and bone-marrow changes. The mesenchymal cells of the bone-marrow showed varying degrees of toxic necrosis, but the blood picture and blood counts were unaltered. From this, we have to presume that the unaffected portion of the bone-marrow is capable of meeting the normal requirements. There was no evidence of increase in the argentophilic reticulum or collagen fibres in the marrow of animals to which repeated small doses of the chemical were given. On reviewing the changes in the liver, spleen and bone-marrow, the liver seems to suffer most and the spleen and bone-marrow to a lesser extent, as judged by ordinary histological criteria. This is but natural, as the liver has the additional function of de-toxication. This experiment may also help to explain why anæmia does not set in early in human beings, in various toxic conditions where the liver, kidneys and spleen show extensive degenerative changes. The bone-marrow seems to be more stable and have reserves to circumvent the damage wrought by various toxins. For this experiment to be more complete, the animals have to be kept under observation for longer periods, some continuing to receive the toxin and others after cessation of the injections. The possibility of the onset of anæmia under these circumstances has to be borne in mind.

The utility of this experiment, in understanding the pathogenesis of Banti's disease, may also be considered. If Banti's syndrome is accepted as a clinical entity, it is preceded by splenomegaly and non-hæmolytic anæmia. Of the very many theories that have been suggested from time to time, two deserve consideration, the first being that the splenic changes are primary, the ætiological factor being shrouded in mystery. The spleen is said to have a regulatory influence on the bone-marrow and also to prolong the life of the red blood cells. Loss of such an influence might result in anæmia. Pearce *et al.* (1918) found that artificially induced anæmia in splenectomized dogs was of greater severity and ran a longer course than in control animals. They attributed this to failure of blood regeneration in the absence of the spleen. Later, Gordon and Kleinberg (1937) experimenting on guinea-pigs have shown that the response of the erythropoietic system is the same in splenectomized and control animals. So, the view that the spleen exerts an influence on the activity of the erythropoietic system does not appear to be substantiated. The second view is that a common toxin acts on the spleen producing splenomegaly, and on the bone-marrow resulting in anæmia. The cirrhosis in the liver may be the result of the same toxin or a secondary product of the diseased spleen reaching the liver through the portal circulation.

Rabbits, injected with small doses of manganese chloride over prolonged periods, develop cirrhosis of the liver and splenomegaly with a histological picture identical with that seen in splenic anæmia. Since anæmia was not noticed at any stage of our experiment, the probability of a common toxin of the nature of manganese chloride in the ætiology of Banti's disease may reasonably be excluded.

CONCLUSIONS.

1. A detailed study of blood and bone-marrow changes in rabbits has been made after administering repeated small doses of manganese chloride.
2. In addition to degenerative changes in the liver and spleen, toxic necrosis of the bone-marrow cells was observed, but of a lesser degree.
3. The hæmoglobin content of the blood and the peripheral blood cell counts were found unaltered, in spite of the damage to the bone-marrow and fibrotic changes in the spleen.
4. Reparative fibrotic changes seen in the liver and the spleen were not in evidence in the bone-marrow.

5. Experimental induction in rabbits, of a clinical condition similar to Banti's syndrome, with a toxic substance of the nature of manganese chloride is not possible, since only hepatic cirrhosis and fibrotic splenomegaly without anaemia were produced.

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HÆMATOLOGICAL STUDIES IN *SILENUS (MACACUS) RHESUS*.

Part I.

THE BLOOD PICTURE OF THE NORMAL MONKEY.

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DURING the course of certain experimental studies on anæmia in *rhesus* monkeys in this laboratory, the lack of reliable normal standards for these animals with which to compare experimental findings was soon felt. A search of the literature revealed few references to the subject but even these few references were incomplete and inadequate, either on account of the technique employed, or the limited number of animals used, or both. Most workers, including Wills and Bilimoria (1932), who have done experimental work on anæmia on monkeys in India, have been content with the estimation of hæmoglobin and the enumeration of red and white cells from the capillary blood of the animals and until recently, when Rao and Rao (1940) published their findings, no references, so far as we could trace, were made to the estimation of the mean corpuscular volume, mean corpuscular hæmoglobin and the mean corpuscular hæmoglobin concentration which are essential to the determination of the type of anæmia. Rao and Rao's work, however, was confined to *sinicus* monkeys and was done at an altitude of 6,000 ft. above sea-level in Coonoor, South India, on a series none too large for the purpose. There is thus need for collecting further data on the subject, especially with reference to *rhesus* monkeys under conditions in which they are generally used for experiments on the plains.

Animals.—Fifty-nine *rhesus* monkeys, weighing from 6 lb. to 14 lb., were used in this study. The exact age of the animals could not be ascertained as they were all wild-caught but none of them appeared very young or very old. The animals were housed in individual cages, and were fed on a diet of grams, fruits and greens, which had been found, from past experience, to be suitable for the maintenance of perfect health.

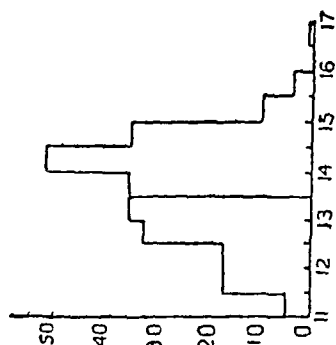
Collection of blood.—All the examinations were made with venous blood which was drawn with a dry syringe from a posterior leg vein. The blood was collected in specially prepared flasks containing a known amount of a mixture of ammonium and potassium oxalates.

Technique.—The samples of blood were examined according to the technique described by Napier and Das Gupta (1942).

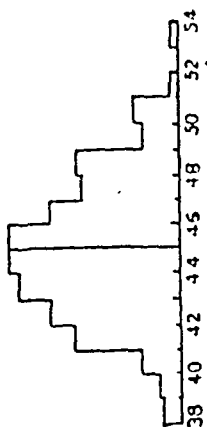
Each sample of blood was examined for hæmoglobin, total red cells, cell volume and reticulocytes and in each case the mean corpuscular volume (MCV), the mean corpuscular hæmoglobin (MCH) and the mean corpuscular hæmoglobin concentration (MCHC) were calculated from the first three data. In all, a total of 245 such examinations were made on 59 monkeys; in 11 monkeys, only one blood-count per animal was done but the remaining 48 were repeatedly examined, usually at weekly intervals, for periods varying from one month to eleven months.

HISTOGRAMS.

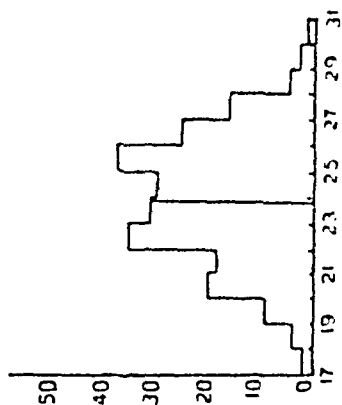
Results of 245 blood examinations on 59 normal monkeys.



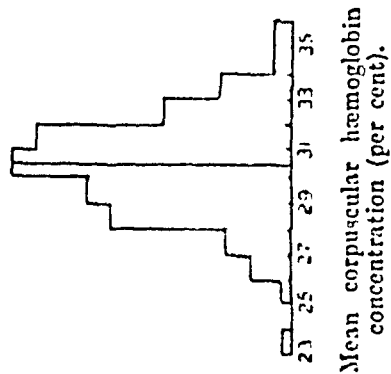
Hemoglobin (g. per 100 c.c.).



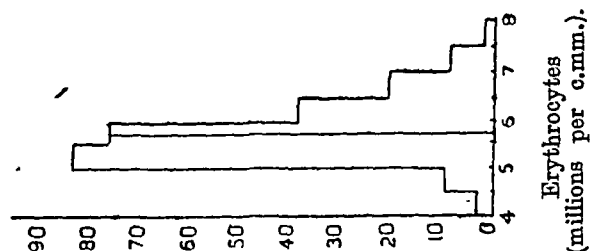
Packed red cell (per cent).



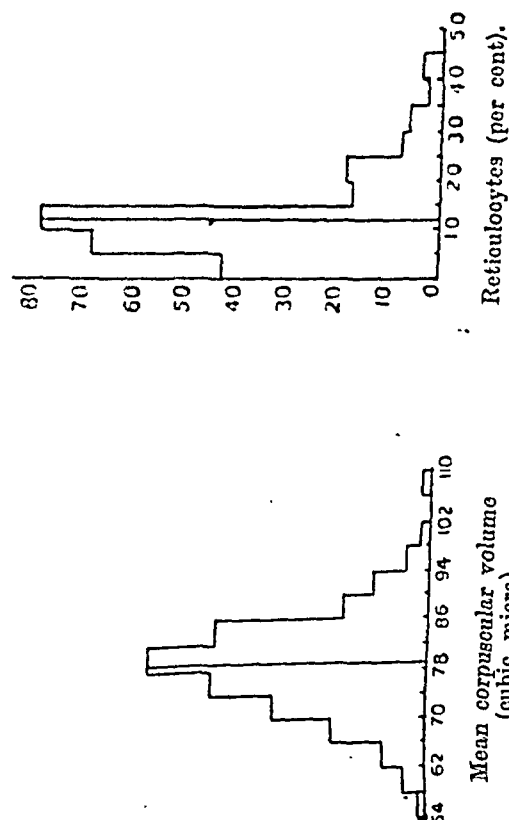
Mean corpuscular hemoglobin (gamma gamma).



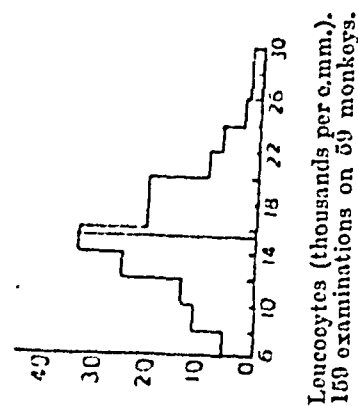
Mean corpuscular hemoglobin concentration (per cent).



Erythrocytes (millions per c.mm.).



Mean corpuscular volume (cubic micra).



Leucocytes (thousands per c.mm.).
159 examinations on 59 monkeys.

TABLE.

PRESENT AUTHORS.					RAO AND RAO (1938).		SHUKERS <i>et al.</i> (1938).		
					<i>M. sinicus.</i>		<i>M. mulatta.</i>		
					(S. rhesus).				
	Number of monkeys.	Number of observation.	Maximum.	Minimum.	Mean.	Standard deviation.	Co-efficient of variation, per cent.	Group A. Group B.	
								Mean.	Mean.
Haemoglobin (g. per 100 c.c.)	59	245	16.78	11.00	13.59	± 1.04	7.65	14.1	12.2
Erythrocytes (millions per c.mm.).	59	245	7.61	4.23	5.76	± 0.63	10.85	6.305	5.20
Cell volume (per cent)	59	245	53.3	38.0	44.98	± 2.69	5.98	43.7	49.7
Mean corpuscular volume (cubic micra).	59	245	106.5	54.9	78.51	± 8.00	10.19	70.54	87.66
Mean corpuscular haemoglobin (gamma gamma).	59	245	30.5	17.7	23.84	± 2.50	10.49	21.09	24.69
Mean corpuscular haemoglobin concentration (per cent).	59	245	35.6	23.8	30.40	± 1.90	6.25	32.54	27.85
Reticulocytes (per cent)	59	245	4.0	0.0	1.19	± 0.81	68.07
Leucocytes (thousand per c.mm.)	59	156	28.0	6.3	15.38.	± 4.30	27.96	16.6	15.0
Neutrophils	34	34	15.618	3.040	7.270	± 4.840	66.57
Lymphocytes	34	34	63.5	38.0	48.3	31.9	36.0
Monocytes	34	34	16.960	2.118	7.210	± 2.770	38.41
Eosinophils	34	34	64.0	31.0	47.9	± 156	64.46	62.0	59.0
Basophils	34	34	522	0.0	242	± 369	112.50	3.2	..
	34	34	4.5	0.0	1.61
	34	34	1,040	0.0	328	2.7	..
	34	34	10.0	0.0	2.2	0.2	..
	34	34	Seen occasionally	

* Values calculated from the available mean data.

Total reticulocyte counts were done 156 times on 59 monkeys while differential leucocyte counts were done on 34 monkeys, once on each animal. The van den Bergh test was done in a number of cases and was found to be below 0.2 mg. per cent in all cases.

Results.—The results of the different hæmatological values are expressed in the Histograms and in the Table. The results obtained by Shukers *et al.* (1938) in *M. mulatta* and by Rao and Rao (*loc. cit.*) in *M. (=S.) sinicus* in the two groups of monkeys A and B are also given in the Table. It may be pointed out here that the animals in group A of Rao and Rao were kept at an altitude of 6,000 ft. for over a year, while the other group had only recently been brought to the hills from the plains.

Comment.—The hæmatological values obtained by us in *rhesus* monkeys are almost identical with the values obtained by Shukers *et al.* (*loc. cit.*) in *M. mulatta** excepting the differential counts of the leucocytes.

Comparing our values in *rhesus* monkeys with those in the two groups of *sinicus* monkeys obtained by Rao and Rao (*loc. cit.*), we find that the hæmoglobin values of both their groups, the red cell count of group B and the corpuscular volume of group A, are almost of the same order as ours, but the red cell count of their group A and the corpuscular volume of their group B are much higher than those obtained by us in *rhesus*. These differences are reflected on the corpuscular values, namely, mean corpuscular volume, mean corpuscular hæmoglobin and mean corpuscular hæmoglobin concentration. Rao and Rao themselves found a significant difference in the red cell counts in the two groups of monkeys A and B belonging to the same species but this difference was probably due to the long residence of group A monkeys at a high altitude. Comparing statistically, a significant difference is observed between the red cell counts of our group of monkeys and of group A monkeys of Rao and Rao, which difference is probably due also to the long residence of group A monkeys at a high altitude. Unfortunately, these workers do not mention the number of observations on which their data regarding corpuscular volume and mean corpuscular values are based and thus it is not possible to say whether the difference between the values obtained by us on *rhesus* monkeys in the plains, and those obtained by Rao and Rao in *sinicus* monkeys at a high altitude, is significant.

The mean value for the total leucocyte count obtained by us in *rhesus* monkeys is slightly lower than the mean value obtained by Rao and Rao, but a very great difference is seen in the differential leucocyte counts in the two series, the lymphocyte count being almost twice as high as the neutrophil count in the A group of monkeys examined by Rao and Rao, while lymphocytes and neutrophils appear in about equal numbers in our series.

SUMMARY.

The hæmatological findings based on a large number of observations in 59 *S. (=M.) rhesus* monkeys are given. A comparison is made with similar findings in *M. mulatta* and *M. sinicus*.

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**Macacus rhesus* and *Macaca mulatta* are synonyms and it is probable that the animals referred to are identical species.—Editor.

ESTIMATION OF STATURE FROM LONG BONES OF PUNJABIS.

BY

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THE estimation of stature from measurements of the long bones is considered notoriously inexact, the error being anything up to five inches (Humphrey, quoted by Modi, 1936). Some British jurists would appear to be reluctant to regard this method of estimation of stature as anything more than mere guesswork (Dixon Mann, quoted by Nat, 1931). Others insist on the measurement of at least half the skeleton for reliable results (Glaister, quoted by Nat, *loc. cit.*).

In India, Pan (1924) at Calcutta measured the long bones of 142 bodies of Bengali Hindus (males and females), and determined the length of each long bone relative to the stature. Later, Nat (*loc. cit.*) at Lucknow published results of similar estimations based on the examination of long bones of 50 residents of the United Provinces. The results obtained from the residents of one province are not necessarily applicable to those of another. This is especially true in the case of Punjabis in whom many factors, natural and artificial, have tended to produce a type different in stature and body-build from other Indians. The present investigation was undertaken to provide records of the length of the long bones relative to body-height of Punjabis. The results obtained promise to be of medico-legal interest, and should be of assistance to medical jurists working in the Punjab.

Unclaimed adult male bodies which had been obtained for dissection purposes in the Anatomy Department of the King Edward Medical College, Lahore, were used in this investigation. Each bone measured was identified as belonging to a body the stature of which had been previously measured. Diseased bones were rejected. The measuring instruments used were Broca's osteometric board, Flower's callipers and the slide compass (Wilder, 1920). All the bones examined were fresh, so that the articular cartilages were always intact.

In all, bones from 110 upper and 110 lower extremities were examined. The results obtained are set out below:—

The length of each bone relative to body-height has been expressed by a 'Multiplication Factor' determined by dividing the average height of the 110 bodies examined (1,636 mm.) by the average length of the long bone in question.

Humerus.—Measurements taken between the lower edge of the trochlea and the highest point of the head of the bone. Average length 321 mm. (longest 352 mm., shortest 226 mm.). The number by which the length of the bone is to be multiplied to get the stature (Multiplication Factor or M.F.) is 5.0, which is got by dividing the average stature (1,636.0 mm.) by the average length of the humerus (321 mm.).

Radius.—Measurements taken: between the highest point on the margin of the head and the lowest point on the styloid process.

Average length = 250 mm. (longest 295 mm., shortest 218 mm.). Multiplication Factor = 6.5.

Ulna.—Measurements taken: between the highest point on the olecranon and the tip of the styloid process.

Average length = 269 mm. (longest 318.5 mm., shortest 230.5 mm.). Multiplication Factor = 6.0.

Femur.—Measurements taken : the actual (and not the apparent) length.

Average length = 445 mm. (longest 522 mm., shortest 395.5 mm.). Multiplication Factor = 3.6.

Tibia.—Measurements taken : between the tip of the medial malleolus and the highest point of the intercondylar eminence.

Average length = 385 mm. (longest 461 mm., shortest 335 mm.). Multiplication Factor = 4.2.

Fibula.—Measurements taken : between the highest point on the head, and the lowest point on the lateral malleolus.

Average length = 373 mm. (longest 453 mm., shortest 324 mm.). Multiplication Factor = 4.4.

The difference between the published Multiplication Factors for Europeans and Indians of various provinces is shown in Table I :—

TABLE I.
Multiplication Factors.

Country.	Humerus.	Radius.	Ulna.	Femur.	Tibia.	Fibula.
Europe (Humphrey, 1936) ..	5.1	7	..	3.6	4	..
India, Calcutta (Pan, 1921) ..	5.3	6.7	6	3.8	4.4	4.4
— Lucknow (Nat, 1931) ..	5.3	6.9	6.3	3.7	4.48	4.4
— Lahore (present authors, 1943)	5	6.3	6	3.6	4.2	4.4

The accuracy of the above Multiplication Factors in estimating the stature by measuring one single long bone of a Punjabi was tested on a series of bones obtained from 20 upper and 20 lower extremities from bodies the stature of which had been previously measured. These long bones were measured, and the stature estimated with the help of the above Multiplication Factors. The difference between the estimated and the actual stature is given in Tables II and III. The average error for the humerus works out to be 30 mm., for the radius 21 mm.; ulna 26 mm.; femur 23 mm.; tibia 21 mm.; and fibula 25 mm. It is clear, therefore, that in estimating the stature of an individual from a single long bone the error in no case is more than 30 mm. or $1\frac{1}{2}$ ". Taking other sources of error into consideration it can be safely stated that the stature can be estimated from the measurement of a single long bone to within an error of $1\frac{1}{2}$ " at the most. The average height of a Punjabi being 1,636 mm., this error is just over 2 per cent.

TABLE II.

Serial number.	Real stature in mm.	Length of humerus in mm.	Estimated stature.	Error.	Length of radius.	Estimated stature.	Error.	Length of ulna.	Estimated stature.	Error.
1	1,487	297	1,485	2	226	1,469	18	248	1,488	1
2	1,500	284	1,420	80	233	1,514	14	249	1,494	6
3	1,500	286	1,430	70	231	1,501	1	240	1,440	60
4	1,600	324	1,622	22	250	1,625	25	266	1,596	4

TABLE II—*concl'd.*

Serial number.	Real stature in mm.	Length of humerus in mm.	Estimated stature.	Error.	Length of radius.	Estimated stature.	Error.	Length of ulna.	Estimated stature.	Error.
5	1,600	320	1,600	0	241.5	1,569	31	266.5	1,599	1
6	1,613	324	1,620	7	255	1,657	44	275	1,650	37
7	1,612.5	325	1,625	12.5	247.5	1,608.5	4	264.5	1,587	25
8	1,622	330	1,650	28	252	1,638	16	262	1,572	50
9	1,625	319	1,595	30	250.5	1,625	0	273.5	1,641.0	16
10	1,625	327	1,635	10	255	1,657	32	277	1,622	3
11	1,625	338	1,690	35	254	1,651	26	277	1,662	37
12	1,637	315	1,575	62	247	1,605	32	262	1,572	65
13	1,637	320	1,600	37	259	1,683	46	280	1,680	43
14	1,637	325	1,625	12	249	1,618	19	269	1,614	23
15	1,637.5	320	1,600	37.5	252.5	1,641	35	272	1,632	5.5
16	1,675	324	1,620	55	255	1,657	18	275	1,650	25
17	1,675	349	1,705	30	259	1,683	8	274.5	1,647	28
18	1,737	337	1,685	22	261	1,696	41	280	1,680	57
19	1,675	335.5	1,677.5	2.5	256.5	1,667	8	276.5	1,659	16
20	1,687.5	330	1,650	37.5	254	1,661	36.5	279	1,674	13.5

TABLE III.

Serial number.	Real stature in mm.	Length of femur in mm.	Estimated stature.	Error.	Length of tibia.	Estimated stature.	Error.	Length of fibula.	Estimated stature.	Error.
1	1,500	412.5	1,485	15	357	1,499	1	345	1,518	18
2	1,500	417	1,501	1	360	1,512	12	350	1,540	40
3	1,512.5	410	1,512	36	371	1,558	46	355	1,562	50
4	1,525	416	1,491	28	364	1,528	3	360	1,584	59
5	1,525	427	1,537	12	359	1,507	18	340	1,496	29
6	1,600	447	1,609	9	367	1,541	59	363	1,597	3
7	1,600	441	1,587	13	380	1,596	4	361.5	1,590	10
8	1,612	451	1,623	11	390	1,638	26	372	1,636	24
9	1,625	453	1,630	5	387	1,625	0	363	1,597	28
10	1,637	450	1,620	17	398	1,671	34	382	1,680	43
11	1,637.5	463	1,668	31	387	1,596	41	361	1,590	47
12	1,650	456	1,641	6	384	1,612	38	371	1,632	18
13	1,650	451	1,623	27	397	1,667	17	378	1,663	13
14	1,675	457	1,645	30	397	1,667	8	382	1,680	5
15	1,675	455	1,638	37	393	1,650	25	378	1,563	12
16	1,700	469	1,652	48	403	1,692	8	389	1,720	20
17	1,700	463.5	1,668	32	402	1,688	12	387	1,702	2
18	1,735	492	1,771	36	407	1,709	26	397	1,746	11
19	1,725	473	1,728	3	419	1,759	34	398	1,751	26
20	1,737	494	1,778	41	411	1,762	25	398	1,751	14

From our above observations a chart (Table IV) has been prepared to serve as a ready reckoner for medico-legal work in the Punjab :—

TABLE IV.

HEIGHT IN		LENGTH OF BONES IN MILLIMETRES.						
Feet.		mm.	Humerus.	Radius.	Ulna.	Femur.	Tibia.	Fibula.
4'-0"	..	1,200	240	184.6	200	333.3	285.7	272.7
4'-1"	..	1,225	245	188.4	204.1	340.2	291.7	278.4
4'-2"	..	1,250	250	192.3	208.3	347.2	297.6	284.0
4'-3"	..	1,275	255	196.1	212.5	354.1	303.5	289.9
4'-4"	..	1,300	260	200	216.6	361.1	309.5	295.4
4'-5"	..	1,325	265	203.8	220.8	368.0	315.4	301.1
4'-6"	..	1,350	270	207.6	225	375	321.4	306.8
4'-7"	..	1,375	275	211.5	229.1	381.9	327.3	312.5
4'-8"	..	1,400	280	215.3	233.3	388.8	333.3	318.1
4'-9"	..	1,425	285	219.2	237.5	395.8	339.2	323.8
4'-10"	..	1,450	290	223	241.6	402.7	345.2	329.5
4'-11"	..	1,475	295	226.9	245.8	409.7	351.1	335.2
5'-0"	..	1,500	300	230.7	250	416.6	357.1	340.9
5'-1"	..	1,525	305	234.6	254.1	423.6	363	346.5
5'-2"	..	1,550	310	238.4	258.3	430.5	369	352.2
5'-3"	..	1,575	315	242.3	262.5	437.5	375	357.9
5'-4"	..	1,600	320	246.1	266.6	444.4	380.9	363.6
5'-5"	..	1,625	325	250	270.8	451.4	386.9	369.3
5'-6"	..	1,650	330	253.8	275.0	458.3	392.8	375
5'-7"	..	1,675	335	257.6	279.1	465.3	398.8	380.6
5'-8"	..	1,700	340	261.5	283.3	472.2	404.7	386.3
5'-9"	..	1,725	345	265.3	287.5	479.2	410.7	392
5'-10"	..	1,750	350	269.2	291.6	486.1	416.6	397.7
5'-11"	..	1,775	355	273	295.8	493.1	422.6	403.4
6'-0"	..	1,800	360	276.9	300	500	428.5	409.0
6'-1"	..	1,825	365	280.7	304.1	507.0	434.5	414.7
6'-2"	..	1,850	370	284.4	308.3	513.8	440.4	420.4
6'-3"	..	1,875	375	288.4	312.5	520.8	446.4	426.1
6'-4"	..	1,900	380	292.3	316.6	527.7	452.3	431.8
6'-5"	..	1,925	385	296.1	320.8	534.7	458.3	437.5
6'-6"	..	1,950	390	300	325	541.6	464.2	443.1

SUMMARY.

Figures to estimate stature from measurement of a single long bone in Punjabis have been worked out after examining the bones from 220 extremities. It is concluded that the stature can be estimated with the help of these figures to within an error of $1\frac{1}{2}$ " at the most, an error which is just over 2 per cent. A chart to serve as a ready reckoner of heights from the measurements of individual long bones has been prepared for use by medico-legal officers in the Punjab.

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VITAMIN B₂ DEFICIENCY AS A CAUSE OF EYE DISEASES IN BENGAL.

BY

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CASES of riboflavin deficiency have been reported from America. In India, cases have been reported from Madras and it was thought that this deficiency might be prevalent in Bengal. The present investigation was undertaken to ascertain whether this deficiency was prevalent in Bengal and, if so, to determine its importance in the ætiology of eye diseases.

Sebrell (1940) reported that Soler in 1791, and Rampoldi in 1885, had stated that inflammation of the cornea, corneal ulcers and opacities occur in cases of pellagra.

During the last 15 years, experimental work on riboflavin deficiency has shown that the following occur:—

- (a) Alopecia of the eyelashes and eyelids, excess of secretion from the conjunctiva, lacrimation, ptosis, ophthalmia (Goldberger and Lillie, 1926; Chick and Roscoe, 1927; Salmons, Hayes and Guerrant, 1928; Sherman and Sandel, 1931; Thacher, Sure and Walker, 1931).
- (b) Opaque eyeballs. anterior interstitial keratitis, superficial keratitis, keratitis and vascularization of the cornea (Salmons, Hayes and Guerrant, *loc. cit.*; Thacher, Sure and Walker, *loc. cit.*; Bourne and Pyke, 1935; Eckardt and Johnson, 1939; Day, Darby and Langston, 1937; Bessey and Wolbach, 1939).
- (c) Cataract (Thacher, Sure and Walker, *loc. cit.*; Bourne and Pyke, *loc. cit.*; Day, Darby and Langston, *loc. cit.*; Sen, Das and Guha, 1935).

The majority of investigators have reported that the incidence of cataract is less than that of other ocular manifestations.

Sebrell and Butler (1939) reported that a diet deficient in riboflavin produces a syndrome in which lesions of the lips (cheilosis) accompanied by a greasy dermatitis around the nasolabial folds were the chief signs. Dermatitis around the eyes and ears was also observed in some cases. These signs disappear with the administration of riboflavin. Sydenstricker, Geeslin, Templeton and Weaver (1939) observed congestion of the sclera with a small amount of serous exudate from the conjunctiva, and photophobia. They made no reference to keratitis and vascularization of the cornea. Pock-Steen (1939) stated that reduced visual acuity in twilight, mydriasis, conjunctival irritation, keratitis and disturbances of accommodation occurred in some of his patients. He attributed these eye conditions partly to riboflavin deficiency and partly to 'histamine toxicosis'. The 'twilight blindness' differed from night-blindness as it improved by the administration of riboflavin and not by vitamin A. He reported that out of 109 patients treated by him, 67 improved after only one milligram of riboflavin given parenterally. Kruse, Sydenstricker, Sebrell and Cleckley (1940, 1940a)

TABLE I.

Author and year.	Syndrome.	Ocular condition.	Location.	Other signs and symptoms.	Treatment.	Deficiency.
Wright (1930)	.. A & B avitaminosis	Failure or dimness of vision.	Sierra Leone.	Angular stomatitis, eczematous skin, disorders of nervous system.	Cured with yeast and cod-liver oil.	Multiple.
Fitzgerald (1932)	.. Avitaminosis B ₂	Assam (India).	Exfoliative glossitis with ulceration at angles of mouth.	Cured with yeast	..
Moore (1934) \ (1937) (1939)	.. Avitaminosis B ₂	Dimness of vision Retrolubular neuritis Optic atrophy	Nigeria	Sore tongue and mouth, itching of the scrotum.	Cured with yeast and marmite.	..
Landon and Pallister (1935)	Avitaminosis B ₂	Dimness of vision Photophobia	Malaya	Eczema of scrotum, glossitis, cheilosis, sub-acute combined degeneration of the cord.	Cured with yeast and marmite.	..
Metivier (1941)	.. Avitaminosis B ₂	1. Retrolubular neuritis Nutritional amblyopia 2. Epithelial corneal dystrophy.	Trinidad	1. Failure of vision 2. Angular stomatitis	1. Cured with yeast and marmite. 2. Riboflavin	1. Multiple. 2. Riboflavin.

investigated 9 cases of clinical 'ariboflavinosis'. All of these cases had cheilosis, 8 had glossitis and 3 had seborrhœic accumulations at the naso-labial folds. The complaints of these patients varied from an itching, burning sensation of the eyes, to mild or even severe photophobia accompanied by dimness of vision in inadequate light, and even to partial blindness. Injection of the conjunctiva of the lids and in the fornix was noted by them. Congestion of the bulbar conjunctiva with marked circumcorneal injection and gross opacities in the cornea were noted in 5 out of 9 cases. These corneal lesions did not respond to nicotinic acid, thiamine, vitamin C or vitamin A, but the administration of riboflavin improved the conditions. These authors made a further investigation later in the same year on 47 patients and confirmed their previous observations. They found that the earliest and most common sign of 'ariboflavinosis' was circumcorneal injection due to congestion and proliferation of the limbic plexus. The authors believed 'that this form of dietary keratitis begins always as a superficial or sub-epithelial vascularization of the cornea, that superficial opacity is apt to follow'. The authors believed further that 'ariboflavinosis is possibly the most prevalent apparently uncomplicated avitaminosis; it is possible also that it is more easily recognized than others on account of the specific lesions of the eye which occur early in the period of deficiency'. Cosgrove and Day (1942) reported successful treatment with riboflavin of several cases of keratitis with vascularization due to various causes. The cases of interstitial keratitis associated with hereditary syphilis responded more rapidly with the administration of riboflavin than when antisyphilitic treatment alone was given. They also further observed that various corneal diseases recovered more rapidly when treated along with riboflavin. Hou (1940) described the treatment of 36 cases of riboflavin deficiency successfully. Aykroyd and Verma (1942) described cases of superficial keratitis associated with angular stomatitis, fissuring of the tongue, burning sensation in the mouth while taking food, seborrhœic dermatitis of the face with multiple comedones, itching and scaliness of the skin of the scrotum, which yielded to treatment with riboflavin.

In other tropical countries, workers have reported the ocular and general manifestations, summarized in Table I, as being due to deficiency of vitamin B₂ (riboflavin).

CLINICAL FINDINGS.

In order to determine the incidence of riboflavin deficiency in Bengal, cases were investigated among the patients attending the Eye Infirmary, Medical College, Calcutta, and in

TABLE II.
*Distribution and signs of riboflavin deficiency among cases seen in Bengal
(excluding Calcutta).*

Places of examinations.	Number of cases examined.	Angular stomatitis.	FISSURING OF TONGUE.		Keratitis.	Vasculariza- tion of cornea.	Angular stomatitis with fissuring of tongue.
			With complaints.	Without complaints.			
Schools ..	1,366	24	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	8
Orphanages ..	263	1	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i> .
Hostels ..	2,033	65	47	45	<i>Nil</i>	<i>Nil</i>	34
Labourers in jute mills.	264	36	<i>Nil</i>	41	<i>Nil</i>	<i>Nil</i>	21
Patients attending the outdoor of travelling eye dispensaries.	566	3	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	2
Villagers ..	852	15	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	23

The chief complaint is burning sensation while taking hot food.

hostels, orphanages, schools and jute mills in and around Calcutta. Field work was carried out at Kishoreganj in the district of Mymensingh in Eastern Bengal and Santipur in the district of Nadia in Central Bengal. In these two places examinations were made of children attending the schools, people living in the surrounding villages, and patients attending the outdoor clinics of the travelling eye dispensaries of the Association for the Prevention of Blindness, Bengal. A total of 27,309 cases were examined of whom 21,965 were seen at the Eye Infirmary, Calcutta (Table III), and 5,344 elsewhere in the province (Table II). Tables II and III summarize the information obtained in regard to the distribution and signs of riboflavin deficiency.

TABLE III.

Analysis of 5,344 cases examined for signs of riboflavin deficiency at the Eye Infirmary, Medical College Hospitals, Calcutta.

Signs.	Number.	Signs.	Number.
Angular stomatitis	7	Keratitis associated with angular stomatitis and lesions of the tongue and scrotum.	4
Angular stomatitis with fissuring of the tongue.	2	Blepharitis and angular conjunctivitis associated with lesions of the tongue and scrotum.	6
Angular stomatitis with lesions of the tongue and scrotum.	5	Corneal ulcer with vascularization ..	1
Corneal ulcer associated with atypical pellagra.	1		
TOTAL ..	15	TOTAL ..	11
GRAND TOTAL ..		26	

A comparative treatment with vitamin A and riboflavin (vitamin B₂) was carried out on two groups, each of 2 cases, having angular stomatitis and other signs. The cases treated with vitamin A (cases 1 and 3 below) showed more rapid and marked improvement than those treated with riboflavin (cases 2 and 4 below). Other illustrative cases are also described.

Case 1.—Hindu male, aged 6 years, complained of watering of both eyes. He had angular stomatitis but no other general or ocular signs. He was given two injections of vitamin A (50,000 units each) at an interval of 4 days. The angular stomatitis cleared up quickly after the second injection.

Case 2.—Hindu male, aged 9 years, complained of watering of both eyes. He had angular stomatitis but no other general or ocular signs. He was given injections of riboflavin (B₂) 1 mg. daily for 7 days. The angular stomatitis cleared up in 8 days.

Case 3.—Hindu male, aged 28 years, complained of a burning sensation in his mouth whilst taking food, and itching of the skin of the scrotum of 3 months' duration. He had no ocular complaints; vision both eyes 6/6. There was marked angular stomatitis, the tongue was fissured and raw and the skin of the scrotum was scaly and very itchy. He was admitted to hospital, and on 17th September, 1942, was given 100,000 units of vitamin A parenterally. The diet of the patient while in hospital contained no milk or any preparation of milk. On 24th September, the angular stomatitis had completely cleared up, the tongue was better and the patient could take food without any burning sensation in the mouth but the fissuring persisted. The skin of the scrotum also showed marked improvement. The patient was discharged from hospital and was advised to take cod-liver oil.

Case 4.—Hindu male, aged 31 years, complained that for about a year he had felt a burning sensation in the mouth while taking food, and that both eyes watered occasionally. Vision both eyes 6/6. He had angular stomatitis with flattening of the papillæ of the tongue on the right side. The skin of the scrotum was normal. He was admitted to hospital on 16th September, 1942, and was given 3 mg. of riboflavin parenterally daily for 5 days. The diet of the patient while in hospital contained no milk or any preparation of milk. On 24th September (8 days later) the angular stomatitis had cleared up to a large extent but there was no improvement in the burning sensation in the mouth whilst taking food. The patient was discharged from hospital and told to attend for treatment.

Case 5.—Hindu boy, aged 10 years, came to the Eye Infirmary, complaining of redness of the right eye with marked sensitiveness to light and diarrhoea of 15 days' duration. He gave a history of having suffered in the past from jaundice, diarrhoea and attacks of inflammation of the eyes. Examination revealed a delicate child with a

long history of bad health and weighing 46 lb. The tongue was coated and the dorsum showed patches of leucoplakia and pigmentation. The pigmentation was also present on the lobules of both ears, on both eyelids, especially the upper ones, and at the angles of the jaws. The liver was just palpable; the spleen was not enlarged; there was no jaundice; the cervical glands were enlarged. The right eye showed marked photophobia and the boy could not open his eyes. On the lower part of the cornea was a deep punched-out ulcer but the margin showed no infiltration, the pupil was active and the tension was normal. The left eye was not involved. On 7th May, 1942, the patient was given parenterally 1 c.c. of vitamin A (100,000 units) and 1 mg. of vitamin B₂ (riboflavin). Two days later the ulcer showed no improvement and a second injection of vitamin A (100,000 units) was given. On 13th May the ulcer still showed no improvement. The patient was admitted to hospital and from 14th to 30th May 12 injections of 50 mg. each of nicotinic acid were given intramuscularly and the pupils were kept dilated by atropine. On 30th May the ulcer had completely healed up, only slight congestion of the conjunctiva and photophobia remained. The pigmentation of the tongue had to a large extent disappeared and the pigmentation in other places had completely disappeared. Vision both eyes was 6/9. The patient was discharged from hospital and was given another six injections of nicotinic acid in the out-patient department.

The above 5 cases were admitted into hospital and kept on the same diet. The following cases of keratitis were diagnosed clinically as due to B₂ (riboflavin) deficiency and were treated as out-patients at the Eye Infirmary:—

Case 6.—Hindu male, aged 60 years, complained of redness of both eyes off and on for 8 months. In both eyes the conjunctiva was congested, circumcorneal injection was present and there were multiple small ulcers which stained with fluoresceine. Superficial vascularization was present all round the periphery of the cornea. Vision R.E.-F.C. at $\frac{1}{2}$ ft., L.E.-F.C. at 2 ft. He was given one dose of 4 mg. vitamin B₂ (riboflavin) parenterally and 4 mg. riboflavin daily by the mouth for 15 days. He returned after 19 days, the ulcers were healed and the conjunctival congestion had disappeared. Vision both eyes 6/60.

Case 7.—Muslim male, aged 32 years, complained of a burning sensation in the mouth while taking food and itching of the scrotum. He also complained of dim vision in both eyes, sensitiveness to light and watering of the eyes. He had angular stomatitis, the lips were fissured, and the skin of the scrotum was scaly and itchy. In the eyes there was diffuse deep keratitis, the corneal sensitivity was normal and the vision of both eyes was 6/60. On 28th September, 1942, he was given vitamin A (100,000 units) parenterally, and on 1st October the dose was repeated. All the symptoms and signs, both general and ocular, remained the same. He was then given vitamin B₂ (riboflavin) parenterally 4 mg. daily for 6 days after which the angular stomatitis, fissuring of the tongue and itching of the scrotum, although still present, were much better than before. The irritability of the eyes became less but the corneal opacities remained the same and the vision was not improved.

Case 8.—Muslim male, aged 19 years, complained of a burning sensation of the mouth while taking food and a burning sensation in both eyes for one month. He had angular stomatitis, fissuring of the tongue, but the skin of the scrotum was normal. In the eyes there were linear and dot-like opacities in the superficial and deep layers of the cornea but no blood vessels were seen. The conjunctivae were normal. Vision R.E. 6/18, L.E. 6/24. He was given vitamin B₂ (riboflavin) 5 mg. daily by the mouth for 8 days, after which period the corneal condition showed considerable improvement, there was no angular stomatitis and the burning sensation in the mouth while taking food had disappeared. The vision also improved to: R.E. 6/9, L.E. 6/12.

Case 9.—Muslim male, aged 22 years, complained of a burning sensation in the mouth while taking food and a burning sensation in the eyes for about a year. He had angular stomatitis and the skin of the scrotum was scaly and itchy. Vision both eyes was 6/9. In the cornea of the right eye there were small dot-like opacities in the upper and inner quadrant but no blood vessels were seen. The patient was given vitamin B₂ (riboflavin) 5 mg. by the mouth daily for 5 days, after which the burning sensation in the mouth and eyes considerably diminished. Unfortunately he did not appear again after 5 days' treatment.

Case 10.—Muslim male, aged 25 years, complained of a burning sensation in the mouth and eyes with watering of the eyes and sensitivity to light for 6 months. He had angular stomatitis, fissuring of the tongue and skin of the scrotum was rough and scaly. In the cornea of both eyes there were small superficial opacities with loops of blood vessels in the upper and outer part. These opacities were discrete and between them the areas were hazy. The rest of the cornea showed a dull corneal reflex with diminished sensitivity. Vision both eyes was 6/36. The patient was given vitamin B₂ (riboflavin) 5 mg. daily by the mouth for 8 days. After this period the opacities disappeared and the cornea in both eyes became clear. The angular stomatitis and the condition of the scrotum considerably improved. Treatment was continued for a further 14 days when the vision of both eyes was 6/6.

DISCUSSION.

The main signs and symptoms in the diagnosis of 'aribo flavinosis' are cheilosis (Sebrell and Butler, *loc. cit.*), the tongue lesions and corneal vascularization (Kruse and his colleagues, *loc. cit.*), but clinical and experimental investigations have shown that these features are not always due to 'aribo flavinosis' and may be due to various other causes. Mechanical causes have been responsible for cheilosis in some cases, such as ill-fitting dentures (Ellenberg and Pollack, 1942), and constant salivation from chewing gum, pan or betel. The cure of cheilosis has been reported after the administration of nicotinic acid, pyridoxin and the entire vitamin B complex (Machela, 1942). Smith and Martin (1940) reported successful treatment of cheilosis associated with pellagra, sprue, coeliac disease and digestive upset with intravenous doses of 20 mg. to 50 mg. of pyridoxin daily. Healing of the fissures at the corners of the mouth occurred in 2 to 5 days. These authors question the contention of Sebrell and Butler that cheilosis is specifically due to riboflavin deficiency.

In our series of cases, two similar groups of patients having cheilosis were treated with vitamin A parenterally and with riboflavin. Improvement was noted in both groups of cases, but those having treatment with vitamin A responded more quickly. The other lesions attributed to riboflavin deficiency are also non-specific. The tongue lesions, red raw tongue with fissures, claimed to be characteristic of riboflavin deficiency, failed to respond to riboflavin, but responded in some cases to pyridoxin and yeast (Machela, *loc. cit.*). Sandstead (1942) showed that corneal vascularization (claimed to be pathognomonic of 'ariboflavinosis' by Kruse and his co-workers) was also non-specific, and was present in from 80 to 95 per cent of children, youths and adults examined in whom this was present without any other signs of riboflavin deficiency. Scarborough (1942) has pointed out that circumcorneal injection, described by Sydenstricker and Kruse (1910) as an early sign of riboflavin deficiency, is also non-specific and was present in about 30 per cent of unselected hospital patients examined by him with no other signs or history of riboflavin deficiency.

It is significant that corneal vascularization has not been observed in clinical experiments on human beings given a diet deficient only in riboflavin, but this was observed in control patients receiving the normal requirements of riboflavin (Boehrer *et al.*, 1943). The writers' experience is that avitaminosis A is by far the most prevalent form of uncomplicated avitaminosis in India and their findings do not support the contention of Sydenstricker and his co-workers that 'ariboflavinosis is the most prevalent, uncomplicated avitaminosis'. The writers have not seen any case of retrobulbar neuritis or optic atrophy which may be attributed to vitamin B₂ deficiency as mentioned by other workers in tropical countries.

SUMMARY.

1. The incidence of riboflavin deficiency in Bengal is low.
2. The occurrence of ocular complications due to riboflavin deficiency is rare.
3. Among the small number of cases of vitamin deficiency with ocular complications observed, three responded to treatment with vitamin A.
4. It would seem that cases of angular stomatitis, with lesions of the tongue and scrotum, are due to deficiency of more than one vitamin.

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THE ESTIMATION OF PYRIDOXINE (VITAMIN B₆) IN FOODS USING RICE-MOTH LARVÆ (*CORCYRA* *CEPHALONICA* ST.).

BY

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PYRIDOXINE (vitamin B₆) is a necessary food factor for the rat and for various lower organisms, e.g. yeast, certain insect larvæ, moulds and bacteria. Spies, Beans and Ashe (1939) and others have shown that it is required by human beings, but little is at present known about its rôle in human nutrition. No doubt future work will throw light on this subject.

Knowledge of the distribution of pyridoxine in foods is not as complete or satisfactory as in the case of other members of the vitamin B complex, such as thiamine, riboflavin and nicotinic acid. Chemical methods of determination have been described by Swaminathan (1940) and Scudi (1941), but have certain disadvantages, notably lack of specificity, and the need for numerous manipulations of large volumes of solution to eliminate interfering substances. Bina, Thomas and Brown (1943) have attempted to overcome these difficulties in a recent modification of Swaminathan's method. Biological methods, using rats, chicks, moulds and bacteria have been employed. According to Williams (1943), 'the rat growth method as applied to meat and meat products constitutes the best method for determining vitamin B₆, although unknown vitamins cannot be ruled out completely'. Micro-biological methods, which have proved successful in the case of other vitamins in the B group, have also been extensively tried. Lacto-bacilli, which are used for estimating other B vitamins, have proved less suitable for estimating pyridoxine, owing to the fact that a considerable proportion of pyridoxine in animal tissues is present in a form, which has been tentatively called 'pseudo-pyridoxine' (Snell, Guirard and Williams, 1942). Pseudo-pyridoxine is nearly a thousand times as effective as pyridoxine itself as a nutrilité for lactic acid bacteria, though both substances have the same growth-promoting effect on yeast, insects and moulds such as *Neurospora*. Siegel, Melnick and Oser (1943) have modified the yeast assay method devised by Williams, Eakin and McMahan (1941) so as to eliminate many of the disadvantages of the original method, while Stokes, Larsen, Woodward and Foster (1943) have developed a new method for the estimation of pyridoxine, based on the growth response of a mutant of the mould, *Neurospora sitophila*. This is an x-ray-induced mutant and requires added pyridoxine for growth.

It is clear that fully satisfactory methods of estimating pyridoxine have not yet been evolved, and that there is room for further work in this field. In the present paper, a method based on the use of the rice-moth larvæ (*Corcyra cephalonica* St.) is described. The author (Sarma, 1943) has already shown that this organism requires pyridoxine for growth and that within certain limits the growth of pyridoxine-deficient larvæ is proportional to the amount of pyridoxine in the diet. Evidence was obtained during the investigation that pseudo-pyridoxine has no greater growth-promoting effect on the deficient larvæ than pyridoxine. By the use of this method, the pyridoxine content of a number of common foods has been estimated. The values are in good agreement with those obtained by Swaminathan's (*loc. cit.*) chemical method and with those given by other workers employing rats as experimental animals.

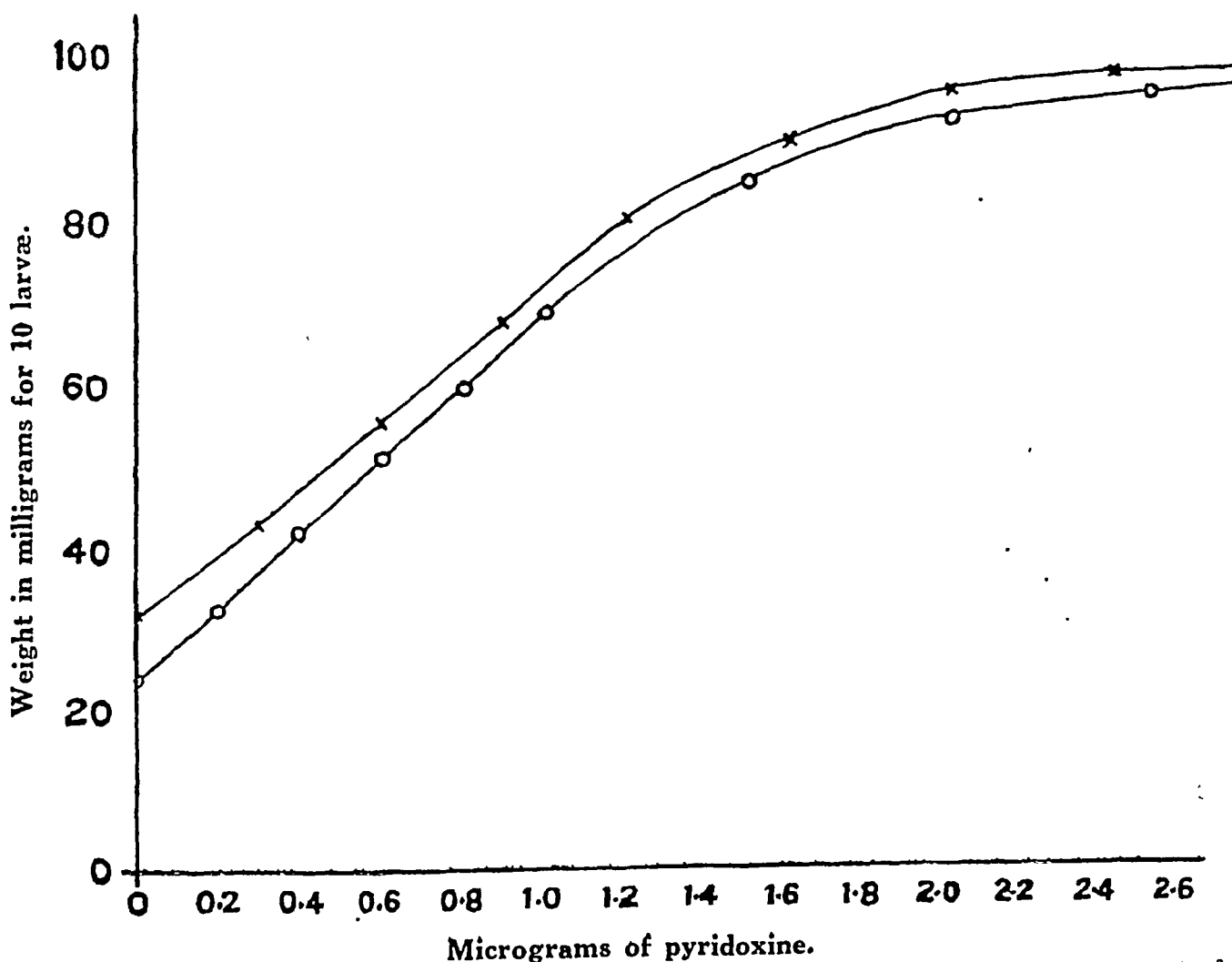
EXPERIMENTAL.

The basal diet was as previously described, i.e. whole-wheat flour extracted with 3 per cent sodium chloride, to which sugar and salt mixture of McCollum and Davis, were added in the proportion of 4 per cent in each case. On this diet, little or no growth occurred with the

addition of B vitamins other than pyridoxine. The pyridoxine-deficient diet was prepared by the addition of thiamine, riboflavin, nicotinic acid and calcium pantothenate, the concentration of these factors per g. of diet being 10 μ g., 5 μ g., 50 μ g. and 15 μ g., respectively. The larvæ, after being given unextracted whole wheat for 8 to 10 days after hatching, were transferred to the pyridoxine-deficient diet. After a depletion period of 28 to 30 days, the pyridoxine tests were begun. At this stage, the weight of 10 larvæ was 25 mg. to 35 mg., very little growth occurring in the absence of pyridoxine. The addition of pyridoxine to the diet caused resumption of growth.

Preliminary experiments were carried out to discover the range within which the increase in growth was proportional to the amount of pyridoxine supplied. Diets were prepared in which known amounts of pure pyridoxine hydrochloride, ranging from 0.2 μ g. to 5.0 μ g. per g. of diet, were included. The Graph shows the growth increase of two groups of pyridoxine-

GRAPH.



Growth response after 14 days of two groups of pyridoxine-deficient larvæ on diets containing varying amounts of pyridoxine.

deficient larvæ after being fed for 14 days on diets containing known amounts of the vitamin. It was deduced from the curves that the best concentration of pyridoxine to work with was between 0.1 μ g. and 1.0 μ g. per g. of diet. Growth response after 14 days was used in evaluating results.

Test materials.—Extracts were prepared by the method of Swaminathan (*loc. cit.*). Pyridoxine exists mainly in the bound form, very little of it being free, so that it is necessary in the first place to liberate it from the bound state and make it water-soluble. Digestion with pepsin appears to liberate the vitamin completely.

The test material was either finely minced or powdered, and a quantity usually varying between 10 g. and 50 g. was taken for pyridoxine assay. The material was suspended in about 150 ml. to 250 ml. of N/10 sulphuric acid, and heated on a water-bath at 70°C. to 80°C. for 30 minutes, with occasional stirring. After cooling to about room temperature, 0.5 g. of pepsin dissolved in 10 ml. of water was added, together with 1 ml. of toluene. The mixture was incubated at 38°C. for 24 hours, after which the solution was again heated on a water-bath at 70°C. to 80°C. for another 30 minutes. The solution was cooled and neutralized with dilute sodium hydroxide solution and centrifuged. The residue was washed with water and again centrifuged and the total centrifugate collected and measured.

Diets were prepared by mixing the extract with the pyridoxine-deficient diet at 2 or 3 different concentrations, the existing knowledge of the pyridoxine content of foods being used to ensure that, as far as possible, the concentration of the vitamin in the test diets would be of the same order as that of the diets containing the pure vitamin. For each assay control diets, usually containing 0.3 µg., 0.6 µg. and 0.9 µg. of pyridoxine per g. of diet, were prepared. Batches of 10 to 15 larvæ, approximately of the same weight, were simultaneously put on the test and control diets. The initial weight of batches varied from 25 mg. to 40 mg. Because of this variation in weight, it was found advisable to run 2 or 3 control tests with each test of food extract.

After the larvæ were weighed, each batch was put in a separate Petri dish. The various diets were sprinkled over them in more than the necessary quantity. The larvæ were kept at 30°C. in an incubator and weighed after 7 and 14 days. Increase in weight after 14 days on various concentrations of extract was compared with increase on the control diets. The values for pyridoxine estimated from the growth response at 2 or 3 different levels of concentration of the extract were averaged to give the final figure.

RESULTS.

Table I gives the results obtained with three foodstuffs, which are typical of the remainder :—

TABLE I.

Pyridoxine content of wheat, tapioca and mango at different assay levels.

Amount of extract in the diet.			Initial weight of 10 larvæ, mg.	Weight of 10 larvæ after 14 days, mg.	Increase in weight, mg.	Pyridoxine per g. of diet, µg.	Pyridoxine per g. of foodstuff, µg.
<i>Whole wheat.</i> —							
Total extract :—174 ml.							
Dosage per 5 g. of diet :	2 ml.	..	24.3	36.8	12.5	0.28	6.09
Do.	3 ml.	..	24.6	45.6	21.0	0.47	6.80
Do.	4 ml.	..	25.0	50.8	25.8	0.58	6.30
<i>Tapioca.</i> —							
Total extract :—180 ml.							
Dosage per 5 g. of diet :	2 ml.	..	27.3	34.5	7.2	0.17	3.06
Do.	3 ml.	..	27.4	37.7	10.3	0.24	2.88
Do.	4 ml.	..	27.6	43.8	16.2	0.38	3.42
<i>Mango (ripe).</i> —							
Total extract :—154 ml.							
Dosage per 5 g. of diet :	2 ml.	..	27.6	42.1	14.5	0.34	2.62
Do.	4 ml.	..	27.2	61.3	34.2	0.78	3.00

It will be seen from Table I that the values for pyridoxine obtained at various concentrations agree fairly closely, indicating that there were no stimulating or inhibiting substances in the various extracts and that the increase in growth was solely due to the amount of pyridoxine present in the diet. It was found that the duplicate values obtained on the same material in different experiments agreed within 15 per cent. Recoveries of pyridoxine added to test materials were quantitative, being in the range of 85 to 100 per cent.

The values for a number of common Indian foodstuffs, and for various other substances, are given in Table II. For the sake of comparison, values obtained by the chemical method of Swaminathan (*loc. cit.*), the rat method of Waisman and Elvehjem (1941), and by various other methods are given in this table. The results obtained are in good agreement with those given by animal assays, on the same or similar biological materials. The values given for some of the test materials by the chemical method are a little on the higher side, which is probably due to extraneous pigments in the extract interfering in the colorimetric determination of the vitamin.

TABLE II.
Pyridoxine content of foodstuffs and biological materials.
Vitamin B₆ hydrochloride in μ g. per g.

Foodstuff.	Larval method.	Chemical method (A).	Rat method.	Other methods.
<i>Cereals.</i> —				
Cambu (<i>Pennisetum typhoides</i>) ..	11.6	10.7
Rice, raw, milled ..	2.3	3.3
Rice, parboiled, milled ..	4.2
Jowar (<i>Andropogon sorghum</i>) ..	7.3	8.0
Ragi (<i>Eleusine coracana</i>) ..	4.3
Whole wheat ..	6.4	8.1	4.6 (C)	4.2 (H) 4.8 (D) 4.51 (G)
Refined wheat flour ..	2.56	3.3	2.2 (C)	1.2 (D) 2.8 (G) 0.9 (H)
<i>Pulses.</i> —				
Bengal gram (<i>Cicer arietinum</i>) ..	11.0	10.9
Green gram (<i>Phaseolus radiatus</i>) ..	11.4	10.1
Soya bean (<i>Glycine hispida</i>) ..	7.9	8.6	..	12.0 (E)
Black gram (<i>Phaseolus mungo</i>) ..	11.3	9.9
<i>Vegetables.</i> —				
Cabbage ..	2.56	2.9
Carrots ..	2.20	1.9
Brinjal (<i>Solanum melongena</i>) ..	1.88
Plantains, raw (<i>Musa paradisiaca</i>) ..	1.36
French beans (<i>Phaseolus vulgaris</i>) ..	0.96
Potato (<i>Solanum tuberosum</i>) ..	2.70	1.6

TABLE II—concl'd.

Foodstuff.	Larval method.	Chemical method (A).	Rat method.	Other methods.
<i>Fruits.—</i>				
Mango, ripe (<i>Mangifera indica</i>) ..	2.81
Orange	1.75
<i>Flesh foods.—</i>				
Sheep liver	6.10	13.8	3.7 (B)	..
Sheep muscle	2.37	4.6	3.0 (B)	4.4 (G)
Sheep kidney	1.65
Sheep heart	1.58
Fish muscle	2.10
<i>Miscellaneous.—</i>				
Whole hen's egg	0.65	0.48 (H)
Cow's milk (per ml.)	1.20	1.8	1.3 (B)	{ 0.5-0.6 (D) 0.71 (G) 0.51 (H)
Torula yeast (<i>Torula utilis</i>) ..	44.1
Brewer's yeast	45.6	51.8	55.0 (F)	{ 39 (D) 65-75 (E) 49 (H)
Ground-nut (<i>Arachis hypogea</i>) ..	7.26
Tapioca (<i>Manihot utilissima</i>) ..	3.12

Key.—

- (A) Swaminathan (1940).
 (B) Waisman and Elvehjem (1941).
 (C) Tepley, Strong and Elvehjem (1942).
 (D) Atkin, Schultz, Williams and Frey (1943).
 (E) Bina, Thomas and Brown (1943).
 (F) Conger and Elvehjem (1941).
 (G) Stokes, Larsen, Woodward and Foster (1943).
 (H) Siegel, Melnick and Oser (1943).

The results show that cereals and pulses are fairly rich in pyridoxine, particularly the latter. Vegetables and fruits are poor sources of pyridoxine, while yeast is a good source. Milk, meat, fish and eggs gave low values, liver being richer than muscle.

The pyridoxine values for sheep muscle, sheep liver, milk and egg obtained by the larval method are in good agreement with those obtained by the rat assay method. This suggests that the 'pseudo-pyridoxine', which is supposed to be present in animal tissues, has no greater growth-promoting effect on the larvæ than pyridoxine itself. This was confirmed when solutions of pyridoxine were hydrolysed with casein and mixed with the diet. No increase occurred in the growth-promoting activity of pyridoxine, as was found to be the case with lactic acid bacteria (Stokes *et al.*, *loc. cit.*).

DISCUSSION.

Pyridoxine deficiency can readily be produced in rice-moth larvæ by giving them a basal diet as described, which includes thiamine, riboflavin, nicotinic acid and calcium pantothenate.

but no pyridoxine. On this diet, little or no growth occurs, but on the addition of pyridoxine growth is as good as that obtained on whole wheat. Pyridoxine values obtained by the larval method agree well with those given by the rat growth method, which is generally considered the most reliable. It is, therefore, considered that the larval method can be safely employed. It has the advantage that it is easy to handle large numbers of larvæ. The rearing and housing of the larvæ in Petri dishes in the incubator is economical as compared with the rearing and housing of rats. A large number of estimations can be carried out in a short space of time. Further, in the methods involving the use of micro-organisms or *Neurospora*, contamination has to be strictly avoided, but the larval method of assay does not require bacteriological control.

The distribution of pyridoxine in foods is interesting. The richness of cereals and pulses suggests that pyridoxine deficiency is unlikely to be common in India, since cereals form the bulk of Indian diets and pulses are consumed in greater quantities than in most other countries. The foods commonly designated as 'protective', e.g. milk, meat, vegetables and fruits, are in fact rather poor sources of this vitamin. Another point of interest is that the loss of pyridoxine occurring on the milling of rice and wheat is considerably smaller than the losses of thiamine or nicotinic acid which result from milling. The more uniform distribution of pyridoxine throughout the grain may be related to the fact that a major portion of it occurs in nature in a bound state.

SUMMARY.

1. A larval method for the estimation of pyridoxine in foodstuffs and biological materials has been developed, based on the growth increase of pyridoxine-deficient rice-moth larvæ (*Corcyra cephalonica* St.).

2. The increase in growth of pyridoxine-deficient larvæ was proportional, within the range of 0.0 μ g. to 1.0 μ g. of pyridoxine per g. of diet, to the amount of vitamin present in the diet.

3. About thirty foodstuffs and biological materials have been analysed for their pyridoxine content by the larval method. The values are in good agreement with those obtained by Swaminathan's chemical method or by various animal assay methods.

4. Cereals and pulses, particularly the latter, are generally fairly rich in pyridoxine. Milk, meat, vegetables and fruits are poor sources of the vitamin.

5. Pseudo-pyridoxine, which is more active than pyridoxine in promoting the growth of lactic acid bacteria, has the same growth-promoting effect on rice-moth larvæ as pyridoxine itself.

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ANTI-THIAMINE FACTOR IN CARP.

BY

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IN another paper (Bhagvat and Devi, 1944, see p. 131, this issue), it has been shown that certain cereals, e.g. ragi (*Eleusine coracana*), and pulses and certain oil-seeds, e.g. mustard (*Brassica juncea*) and linseed (*Linum usitatissimum*), contain two factors which rapidly inactivate thiamine. One factor was found to be thermolabile and non-dialysable, and the other thermostable and dialysable. The existence of a 'thiamine-inactivating' mechanism in carp and certain other varieties of fish was reported by Green, Carlson and Evans (1941, 1942), Woolley (1941), Spitzer, Coombes, Elvehjem and Wisnicky (1941), Evans, Green and Carlson (1942), Green, Evans, Carlson and Swale (1942), Deutsch and Ott (1942) and Deutsch and Hasler (1943), and it was felt to be of interest to compare the fish factor with those present in other foods. With this object in view the present investigation was undertaken. While, however, it was in progress, two papers appeared: one by Sealock, Livermore and Evans (1943) and the other by Krampitz and Woolley (1944). The former authors studied the properties of the fish anti-thiamine factor, while the latter in addition, isolated the products of breakdown of thiamine formed by the action of carp tissues. Krampitz and Woolley found that the destruction of thiamine by carp tissue was an enzymic reaction and the enzyme was found to consist of two components, one heat-labile and non-dialysable and the other heat-stable and dialysable. Either component alone had some slight activity against thiamine, but the two together were necessary for full activity. In this respect, the anti-thiamine factor from ragi and oil-seeds appears to be different from the carp factor. The results presented in this paper are, in general, in agreement with those reported by Sealock *et al.* and Krampitz and Woolley.

EXPERIMENTAL.

Extraction of the anti-thiamine factor from carp tissues.—A freshly caught carp was killed by decapitation and the blood was collected in 5 per cent sodium acetate solution. Usually 3 ml. of blood were obtained from a medium-sized carp ($\frac{1}{2}$ lb.), which was made up to 50 ml. with 5 per cent sodium acetate solution. The solution was filtered and a suitable aliquot of the clear filtrate was used for testing its activity against thiamine.

The muscle and viscera were extracted with the following solvents: (i) water, (ii) 10 per cent NaCl and (iii) chloroform-water mixture (5 ml. chloroform in 95 ml. of water). The tissues (20 g.) were ground up finely with 100 ml. of each of the solvents and the suspensions thus obtained were left overnight at 0°C. with toluene as a preservative. In the case of the chloroform-water mixture it was found by preliminary experiments that almost complete extraction of the factor was achieved, when the tissues were ground up with this solvent only for 5 to 10 minutes. The different suspensions were filtered and suitable aliquots of the clear filtrates were tested for their anti-thiamine potency.

Known aliquots of the different extracts and diluted blood were brought to pH 5.6 with M/2 acetate buffer and were incubated overnight at 37°C. with 25 μ g. of thiamine. The amount of the vitamin left over in the different extracts was estimated by the thiochrome method as described by Bhagvat (1943). A parallel incubation with 25 μ g. thiamine was carried out with the different extracts after they were boiled for 2 to 3 minutes.

The results are given in Table I :—

TABLE I.
Inactivation of thiamine by carp muscle, viscera and blood.

			μg. thiamine destroyed.
<i>Muscle.</i> —			
1. Aqueous extract	.. {	Unboiled	14.0
		Boiled	0.3
2. 10 per cent NaCl	.. {	Unboiled	22.4
		Boiled	5.0
3. Chloroform water	.. {	Unboiled	25.0
		Boiled	0.0
<i>Viscera.</i> —			
1. 10 per cent NaCl	.. {	Unboiled	25.0
		Boiled	2.6
2. Chloroform water	.. {	Unboiled	25.0
		Boiled	2.6
Blood	.. {	Unboiled	25.0
		Boiled	4.3

It is clear from Table I that (i) carp tissues and blood contained a factor which inactivates thiamine, (ii) the factor, as in the case of ragi and oil-seeds, is soluble in chloroform-water mixture and (iii) it is thermolabile, thereby indicating its enzymic nature. Further evidence revealing its enzymic nature was brought forward by the study of the kinetics of the reaction, the relation between the concentration of the factor and the concentration of thiamine, and effect of pH, etc. on the inactivation of thiamine.

Inactivation of thiamine by graded amounts of the viscera and muscle extracts and diluted blood.—This problem was next investigated. The reaction mixture consisted of \times ml. of extracts or diluted blood + 10 ml. of M/2 acetate buffer (pH 5.6) + 25 μg. thiamine. The final volume was made up to 25 ml. The solutions were incubated overnight for about 18 hours at 37°C. Table II represents the results obtained :—

TABLE II.
Inactivation of thiamine by graded amounts of the extracts and diluted blood.

ml. of extract.	μg. thiamine destroyed.
<i>Muscle extract.</i> —	
1.0	0.0
2.5	4.5
5.0	5.8
10.0	18.3
12.5	24.1

TABLE II—concl'd.

ml. of extract.	μ g. thiamine destroyed.
<i>Visceral extract.</i> —	
0.2	15.2
0.3	16.0
0.5	17.9
1.0	25.0
<i>Diluted blood.</i> —	
0.3	21.2
0.5	23.7
1.0	25.0

The amount of thiamine inactivated appeared to depend upon the amount of the extracts taken. Maximum inactivation was obtained with 12.5 ml. of muscle extract, and 1 ml. each of visceral extract and diluted blood.

Rate of destruction of thiamine.—The rate of destruction of thiamine by the extracts and diluted blood at 37°C., pH 5.6 was investigated. At known intervals of time, 25 ml. of the reaction mixture (containing 25 μ g. of thiamine) were pipetted out and the amount of thiamine present was estimated. The results are given in Table III :—

TABLE III.

Rate of destruction of thiamine.

Time in minutes.	μ g. THIAMINE DESTROYED.		
	Muscle extract.	Visceral extract.	Diluted blood.
0	0.0	11.8	..
15	..	18.1	..
30	..	24.2	..
60	10.0	25.0	25.0
120	14.7	25.0	25.0
180	18.4
24 hours	23.2	25.0	25.0

The rate of destruction of thiamine by the extracts and diluted blood increased with time. Maximum inactivation of the vitamin by the muscle extract was observed after 24 hours' incubation, while the same result was obtained with visceral extract and blood after an hour's incubation. Thus, in all subsequent experiments, the incubation of thiamine with muscle extract was carried out for 18 to 24 hours and with visceral extract and blood only for an hour.

Effect of pH on the inactivation of thiamine.—The inactivation of thiamine by carp tissues and blood was carried out between pH 3 and 7.0 using M/2 acetate buffer. pH 3 was obtained by addition of M/2 acetic acid and pH 7 by using M/2 sodium acetate solution. The

inactivation of thiamine appeared (Table IV) to be only slightly affected by changes in pH from 4 to 7.0:—

TABLE IV.

Effect of pH on the inactivation of thiamine.

Reaction mixture	..	{	12.5 ml. of muscle extract	}	10 ml. buffer +
			1.0 " " visceral extract		25 µg. thiamine.
			1.0 " " diluted blood	}	Final vol. 25 ml.

pH.	µg. THIAMINE DESTROYED.		
	Muscle extract.	Visceral extract.	Diluted blood.
3.0	11.8	21.0	..
4.0	17.4	20.4	19.2
4.5	20.3
5.0	23.4	23.7	25.0
5.6	23.5	24.4	25.0
6.0	25.0	25.0	25.0
7.0	25.0	25.0	23.7

Effect of graded concentrations of thiamine.—The relation between the concentration of thiamine and the rate of its destruction at pH 5.6 and 37°C. were next studied. The reaction mixture contained the same amounts of the extracts and diluted blood as used in the previous experiments. The results are given in Table V:—

TABLE V.

Relation between the concentration of thiamine and the rate of its destruction.

µg. thiamine added.	µg. THIAMINE DESTROYED.		
	Muscle.	Viscera.	Blood.
25.0	25.0	25.0	25.0
50.0	48.4	48.0	32.0
75.0	..	65.0	52.0
100.0	74.2	82.0	70.0
125.0	..	101.0	84.0

The rate at which thiamine inactivated was directly proportional to its concentration, when the concentration was low (up to 50 µg.). At higher concentrations the rate of reaction and the concentration were not proportional. When the results were expressed on the dry weight basis, it was observed that 1 mg. of muscle, viscera and blood respectively will inactivate 0.32 µg., 8.6 µg. and 8.3 µg. respectively of thiamine in the hour at 37°C. Thus, the viscera and blood are about 26 times richer in this factor than the muscle. All the results so far presented appear to point to the enzymic nature of the thiamine-inactivating mechanism.

Purification of the enzyme.—(1) *Dialysis of the visceral and muscle extracts.*—The two extracts were dialysed at room temperature (20°C. to 25°C.) for 48·72 hours against running water. The non-dialysable portions were then tested for their activities towards thiamine (Table VI):—

TABLE VI.

Destruction of thiamine by the dialysed extracts of muscle and viscera.

Extract.		PER CENT THIAMINE DESTROYED.	
		Original extract.	Dialysed extract.
Muscle	100·0	31·8
Viscera	95·2	64·7

The extracts appeared to lose part of their activity on dialysis. This might be due to the loss, during dialysis of a catalyst or a co-enzyme, in which case the activity of the extract should be restored on addition of the boiled original extract. Results presented in Table VII show that this occurred:—

TABLE VII.

Extract.		μg. THIAMINE DESTROYED.			
		ORIGINAL EXTRACT.		Dialysed extract.	Dialysed extract + boiled original extract.
		Unboiled.	Boiled.		
Muscle	..	25·0	0·0	8·0	25·0
Viscera	..	23·8	2·4	16·8	23·5

While these experiments were in progress, a paper by Krampitz and Woolley (*loc. cit.*) appeared, wherein the authors have shown that, on dialysis, the enzyme could be resolved into two components: one heat-labile and non-dialysable and the other heat-stable and dialysable. Both the components were necessary for the full activity of the enzyme. Our experiments point to the same conclusion.

(2) *Precipitation with ammonium sulphate.*—Muscle extract was used in this experiment. The extract was treated with graded amounts of ammonium sulphate and it was observed that the enzyme was precipitated only when the extract was fully saturated with the salt. The precipitate was removed by filtration and dissolved in small amount of water. The solution was filtered and the residue was washed with water. The filtrate and the washings were mixed and made up to the original volume of the extract. An aliquot of the solution was dialysed against running water till it was free from ammonium sulphate. The original extract and the solution of ammonium sulphate precipitate before and after dialysis

were tested for their anti-thiamine potency with 25 μ g. thiamine. The results are shown in Table VIII :—

TABLE VIII.

Anti-thiamine potency of original extract and ammonium sulphate precipitate of the enzyme.

						μ g. thiamine destroyed.
Original extract	Unboiled	25.0
					Boiled	5.4
Ammonium sulphate precipitate in H ₂ O (before dialysis)	..				Unboiled	25.0
					Boiled	4.7
"	"	"	"	(after dialysis)	Unboiled	1.8

The enzyme was precipitated by full saturation of the extract with ammonium sulphate. The precipitate, however, lost all its anti-thiamine activity on dialysis. This again suggests the possibility of the enzyme being made up of two components.

(3) *Precipitation with acetone.*—The enzyme was precipitated from the muscle extract by the addition of 3 volumes of ice-cold acetone (this was used in order to ensure minimum loss of activity of the enzyme). The precipitate was removed on the centrifuge and washed repeatedly with ice-cold acetone. It was finally suspended in 5 per cent NaCl. This suspension when tested for its potency was found to contain only half the activity of the original extract. The acetone treatment was then repeated with whole muscle in order to see if a more potent preparation could be obtained. The muscle was finely ground and stirred with 3 volumes of ice-cold acetone. The residue was separated by filtration under suction, washed repeatedly with acetone and dried *in vacuo* over sulphuric acid. Immediately on drying the residue was tested and was found to retain the full activity of the fresh muscle. However, on keeping a progressive loss of activity occurred.

Stability of the enzyme.—That the enzyme was sensitive to heat was shown in experiments reported earlier in this paper. The sensitivity of the enzyme towards acid and alkaline pH was next tested. The muscle extract was brought to pH 10.0 and left overnight. Another portion of the extract was kept overnight at pH 2.0. An extract at pH 5.6 served as a control. The different extracts were adjusted to pH 5.6 and tested for their activity towards thiamine (25 μ g.). Table IX gives the results of a typical experiment :—

TABLE IX.

Stability of the enzyme at pH 2, 5.6 and 10.

pH.	μ g. thiamine destroyed.
2.0	2.5
5.6	25.0
10.0	5.0

The enzyme is thus destroyed by keeping at pH 2 and 10.

The activity of the enzyme was found to be unaffected in presence of inhibitors like potassium cyanide and sodium fluoride in M/100 concentration.

Destruction of thiamine in yeast and animal tissues by the muscle extract.—In the next paper (Bhagvat and Devi, *loc. cit.*) destruction of thiamine in yeast and liver by the extracts of ragi and mustard seeds has been described. It was felt to be of interest to find out whether the muscle extract will show a similar behaviour. Dried brewer's yeast and animal tissues were incubated overnight at 37°C. with 10 ml. of muscle extract. Parallel incubations were carried out with (i) yeast and animal tissues + thiamine and (ii) same as (1) + 10 ml. of muscle extracts. The amounts of the vitamin remaining in the different solutions are shown in Table X. The results show the destructive effect of carp muscle extract under these experimental conditions.

TABLE X.

Destruction of thiamine in yeast and animal tissues by muscle extract.

Materials.	μg. THIAMINE.		Per cent. thiamine destroyed.
	Found.	Calculated.	

Brewer's yeast.—

0.5 g. yeast	31.0
10 ml. muscle extract	0.0	0.0	..
0.5 g. yeast + 10 ml. muscle extract	0.0	34.0	100.0
0.5 g. yeast + 25 μg. thiamine	51.4	59.0	..
0.5 g. yeast + 25 μg. thiamine + 10 ml. muscle extract.	1.1	59.0	97.7

Sheep liver.—

5 g. liver	10.0
5 g. liver + 10 ml. muscle extract	0.0	10.0	100.0
5 g. liver + 25 μg. thiamine	27.0	35.0	..
5 g. liver + 25 μg. thiamine + 10 ml. muscle extract.	1.7	35.0	95.2

Sheep heart.—

5 g. heart	44.0
5 g. heart + 10 ml. muscle extract	0.6	44	98.5
5 g. heart + 25 μg. thiamine	61.5	69	..
5 g. heart + 25 μg. thiamine + 10 ml. muscle extract.	1.8	69.0	94.7

Comparison between the anti-thiamine factor in carp with the factor present in ragi and mustard seeds.—There are certain points of similarity between the two factors: (a) Like carp tissue, ragi and mustard seeds destroy thiamine. (b) 4 methyl-5 hydroxy-ethyl thiazole and 2 methyl-4 amino-5 hydroxy methyl pyrimidine have been isolated as the products of breakdown of thiamine by the action of carp tissue. These have been shown to promote the growth of the larvæ of the mosquito [*Aedes (stegomyia) albopictus*]. Ragi and mustard seeds also appear to break thiamine down into such products, which were shown to be utilized by the larvæ. As regards the nature of these products nothing is known at present, but attempts are now being made to isolate them. (c) The activity of the carp factor, like that of the factor

in ragi and mustard seeds, is unaffected by changes in pH from 3 to 7. (d) Both the factors destroy thiamine in yeast and animal tissues.

While the carp and ragi and mustard seed factors show certain similarities in some respects they exhibit remarkable differences: (a) The carp factor behaves like a typical enzyme. It is thermolabile and its activity depends upon factors which normally affect enzyme activity. The ragi and mustard seed factor does not behave like an enzyme. (b) The carp factor consists of thermolabile and non-dialysable, and thermostable and dialysable, components. Either fraction alone has some slight activity but the two together are necessary for the full activity. The mustard seed factor also consists of two components—heat-labile and non-dialysable, and heat-stable and dialysable. But in this case either fraction by itself was found to be active. (c) The carp factor is destroyed when kept overnight at pH 2 and pH 10; while the ragi and mustard seed factor is perfectly stable under similar conditions.

SUMMARY.

1. Carp muscle, viscera and blood were found to contain a factor which inactivated thiamine and which was extracted by chloroform-water mixture.

2. The factor has been found to behave like a typical enzyme in being thermolabile, its activity being dependent on the relative concentrations of the enzyme and the substrate thiamine.

3. Visceral extract and blood were found to be about 26 times richer in this enzyme than muscle extract.

4. The visceral and muscle extracts have lost their activity after dialysis for 48 to 72 hours. Activity could be restored by the addition of the boiled original extracts—suggesting the presence of two fractions, one heat-labile and non-dialysable and the other heat-stable and dialysable.

5. The enzyme was precipitated by full saturation of the muscle extract with ammonium sulphate. On dialysis, the precipitate was found to lose all its activity. The enzyme was also precipitated by 3 volumes of acetone, but this treatment was found to yield an unstable preparation of the enzyme.

6. The enzyme has been found to destroy thiamine present in dried yeast and animal tissues.

7. The carp enzyme is different from the anti-thiamine factor of ragi and mustard seed though certain similarities between them have been observed.

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INACTIVATION OF THIAMINE BY CERTAIN FOODSTUFFS AND OIL SEEDS.

Part I.

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DURING the course of an investigation on the liberation of thiamine and nicotinic acid from foodstuffs by pig's mucosa enzyme preparation, the disappearance of thiamine on its addition to certain foodstuffs was demonstrated by the application of the thiochrome test (Bhagvat, 1943). The factor responsible for this disappearance was shown to be insoluble in ether and, in the case of rice polishings, thermolabile. The factor in rice polishings appears to share the latter property with the anti-thiamine factor in carp tissues, which was shown by Woolley (1941) and Spitzer, Coombes, Elvehjem and Wisnicky (1941) to inactivate thiamine *in vitro*, the degree of inactivation being reduced on cooking the tissues. The analogy between the two thiamine inactivating mechanisms from two entirely different sources, led us to undertake the present investigation.

EXPERIMENTAL.

The observation of Bhagvat (*loc. cit.*) regarding the low recovery of thiamine added to 3 cereal substances, viz. ragi (*Eleusine coracana*), rice polishings and bajra (*Pennisetum typhoideum*) was confirmed. In addition to these foods a number of others were tested to see if they behave in the same way. A known amount of the material was suspended in 50 ml. of M/10 acetate buffer (pH 5.6), 50 µg. of thiamine were added, and the suspensions incubated overnight at 37°C., with toluene as a preservative. The amount of thiamine remaining in the extracts was estimated according to Bhagvat's method. The results were given in Table I:—

TABLE I.

Percentage recovery of thiamine added to different foodstuffs and oil seeds.

Material.	Botanical name.	Amount taken, g.	Thiamine added, µg.	Percentage recovery of added thiamine.
Ragi <i>Elcusine coracana</i>	5.0	50.0	14.3
Rice polishings	5.0	50.0	28.8
Bajra <i>Pennisctum typhoideum</i>	10.0	50.0	57.7
Wheat germ	5.0	50.0	43.1
Green gram	.. <i>Phaseolus radiatus</i>	5.0	50.0	31.3
Soya bean	.. <i>Glycine hispida</i>	5.0	50.0	60.0
Cow pea <i>Vigna catiang</i>	5.0	50.0	62.0

TABLE I-*concl'd.*

Material.	Botanical name.	Amount taken, g.	Thiamine added, µg.	Percentage recovery of added thiamine.
Bengal gram ..	<i>Cicer aristinum</i>	10.0	50.0	86.5
Mustard seed ..	<i>Brassica juncea</i>	5.0	50.0	25.0
Cotton seed (yellow) ..	<i>Gossypium</i> sp.	5.0	50.0	25.0
Cotton seed	5.0	50.0	8.7
Linseed ..	<i>Linum usitatissimum</i>	5.0	50.0	28.0
Sesamum seed ..	<i>Sesamum indicum</i>	5.0	..	70.0

Table I shows that low recovery of added thiamine was obtained with a number of food-stuffs other than cereals. The oil seeds, mustard and cotton seeds were chosen because (i) of the apparent connection between mustard oil and epidemic dropsy in Bengal and the fact that certain workers have held the view that there is an association between epidemic dropsy and thiamine deficiency, and (ii) because a condition resembling human beri-beri was observed by Rommel and Vedder (1927) (quoted by Williams, 1929) in swine fed on cotton-seed.

The rate of disappearance of thiamine on the addition of various foods was next studied. The amounts of the materials and that of thiamine, and the other experimental conditions, were the same as in the previous experiment. Table II shows the results:—

TABLE II.
Rate of disappearance of thiamine.

PERCENTAGE OF THIAMINE DISAPPEARING.						
Materials.			Time in hours.			
		0 (7 minutes)	1	2	3	24
Ragi	75.0	89.2	81.2	75.0	92.3
Rice polishings	50.0	52.4	59.4	..	68.7
Green gram	76.0	67.2	70.3	62.5	68.7
Mustard seed	64.7	61.8	70.6	64.7	70.6
Cotton seed	75.0	95.6	98.5	100.0	94.1
Linseed	50.0	60.7	64.3	71.4	78.6

The disappearance of thiamine appears to begin immediately after its addition to the test materials. This suggests a non-enzymic reaction. Disappearance might be due to its adsorption on the solid residue. This, however, was not the case as was shown by experiment using a number of elutriants in which thiamine is freely soluble. Thiamine could not be eluted by any of them.

Further, the filtrates, when tested with mosquito larvæ [*Aedes (stegomyia) albopictus*], were found to promote the growth of the mosquito larvæ (detailed description of these experiments is given in Part II of this paper (see p. 139, this issue). Thiamine is one of the essential nutrients for the proper growth of these larvæ, but it was found that thiamine

could be replaced by some of its breakdown products present in the filtrate. Further evidence has been obtained by biological experiments with rats and pigeons to show that disappearance of thiamine is due to its conversion into biologically inactive (for rats and pigeons) components by the test materials.

Another possibility to account for the disappearance of thiamine on its addition to food-stuffs was combination with a specific protein in a manner similar to the inactivation of biotin by avidin (Eakin, Snell and Williams, 1941 ; Woolley and Longworth, 1942).

Next an attempt was made to find out whether the factor responsible for the inactivation of thiamine is thermolabile. Portions (2.5 g.) of the test materials were weighed into 3 flasks containing 25 ml. of M/10 acetate buffer (pH 5.6). One flask was boiled for 15 to 20 minutes, another was autoclaved for 15 minutes at 15-lb. pressure, while the third remained unheated. After the addition of 25 μ g. of thiamine, the flasks were incubated overnight at 37°C. and the amount of thiamine inactivated was estimated (Table III) :—

TABLE III.
*Effect of boiling and autoclaving on the inactivation of
thiamine by aqueous suspensions
of foodstuffs.*

Material.			Percentage of thiamine inactivated.
Ragi ..	{	Unheated	86.4
		Boiled	87.2
		Autoclaved	93.0
Rice polishings ..	{	Unheated	71.0
		Boiled $\frac{1}{2}$ hr.	29.0
		Boiled 1 hr.	13.5
Green gram ..	{	Unheated	81.0
		Boiled	78.0
		Autoclaved	94.1
Mustard seed ..	{	Unheated	80.2
		Boiled	80.2
		Autoclaved	93.0
Cotton seed (yellow) ..	{	Unheated	79.0
		Boiled	78.2
		Autoclaved	53.0
Cotton seed ..	{	Unheated	90.2
		Autoclaved	93.7
Linseed ..	{	Unheated	76.5
		Boiled	80.3
		Autoclaved	94.1

The thiamine inactivating mechanism in the various materials tested, except in those of rice polishings and yellow cotton seed, appeared to be unaffected by heat. This ruled out the possibility of the reaction being enzymic in nature. Further proofs of the non-enzymic nature of the reaction was obtained by the study of (i) the effect of pH and (ii) the relation between the concentration of the material and thiamine. (For these experiments only ragi and mustard seed were used.) The inactivation of thiamine was found to be independent of pH between 3 and 7, and of the amount of the material, amounts between 0.1 g. and 2.5 g. were found to inactivate the same quantity of thiamine. It was observed that if thiamine was ground up with powdered ragi, 2.5 g. of the powder will inactivate 1.2 mg. of the vitamin.

Extraction of the thiamine inactivating factor from ragi.—A number of solvents, given in Table IV, were employed for the trial extraction of the anti-thiamine factor from ragi :—

TABLE IV.

Extraction of the anti-thiamine factor from ragi.

Extracts equivalent to 2.5 g. of ragi + 25 ml. acetate buffer + 25 µg. thiamine.

Solvent.	Period of extraction in hours.	Percentage thiamine inactivated by the extracts.
Water	24-48	70.0
1 per cent sodium chloride soln.	12.2
10	7.8
10 .. potassium chloride soln.	26.0
5 .. sodium acetate soln.	6.1
0.2 M acetic acid	23.1
5 per cent alcohol	28.8
20	28.8
70	36.5
80	40.0
Acetone	11.4
Chloroform-water mixture	2 minutes	94.3

The factor appears to be soluble in water, but insoluble in solutions of salts, viz. sodium chloride, potassium chloride, sodium acetate, dilute acetic acid, alcohol of graded strength and acetone. However, it could be easily extracted on grinding the powdered ragi with chloroform-water mixture (5 ml. of chloroform + 95 ml. of water) for only 2 minutes. In all subsequent experiments, this solvent was used for the extraction of the factor from different test materials. That such extracts were active is shown by the results given in Table V. The effect of boiling and autoclaving (at 15-lb. pressure for 15 minutes) the extracts on their thiamine inactivating potency was also investigated :—

TABLE V.

Inactivation of thiamine by the chloroform-water mixture extracts of different test materials.

Materials.	PERCENTAGE THIAMINE INACTIVATED BY		
	Original extract.	Boiled extract.	Autoclaved extract.
Ragi	94.7	94.7	68.4
Mustard	78.9	71.1	63.2
Cotton seed (yellow)	59.5	28.6	33.3
Cotton seed	92.3	92.4	..
Linseed	24.1	24.1	..

All the extracts were found to be active and their activity with the exception of that of cotton seed extract appeared to be unaffected by boiling or autoclaving.

Effect of dialysing the chloroform-water mixture (C. W.) extracts of different materials on their thiamine inactivating potency.—The C. W. extracts of different test materials were dialysed for various lengths of time at room temperature (25°C.) in cellophane bags. The undialysable portion was tested for its activity towards thiamine (Table VI) :—

TABLE VI.

Effect of dialysis of the C. W. extracts on their thiamine inactivating potency.

Materials.	Dialysis for	PERCENTAGE THIAMINE INACTIVATED.				
		ORIGINAL EXTRACT.		DIALYSED EXTRACT.		
		Unboiled.	Boiled (for 1 min.).	Unboiled.	Boiled for 1 min.	Autoclaved 15 min. at 15 lb.
Ragi ..	24 hours	83.0	..	26.2	33.2	..
	48 ..	78.6	71.4	28.6	17.9	..
Mustard seed ..	24 ..	59.7	52.8	76.4	75.0	..
	48 ..	70.6	70.6	76.5	80.0	32
	96 ..	91.7	..	50.0	18.7	..
Linseed ..	48 ..	36.7	35.2	90.0	46.7	40.0
Cotton seed (yellow)	48 ..	53.8	23.8	99.7	38.5	17.9
Green gram ..	48 ..	20.3 *	14.9	76.5	35.3	..

The C. W. extract of ragi appeared to lose part of its activity on dialysis, while the extracts of other materials retained their activity, which was partially lost when the dialysed extracts were boiled or autoclaved. This suggests the presence of two factors, one being thermostable and dialysable and the other thermolabile and non-dialysable. Further, the activity appears to reside in both the fractions, each of which inactivates thiamine independently. Further confirmation of this was obtained by testing the anti-thiamine potency of the dialysates from ragi and mustard extracts. Both the dialysates were active and retained their activity even on boiling. Ragi, however, contains predominantly the thermostable factor.

The activity towards thiamine of the undialysed C. W. extracts of the different test materials was found to be independent of pH between 3 and 7, of the amount of the extracts, and the amount of thiamine used for the test. The activity of the undialysable portion of the mustard extract, however, though unaffected by changes in pH from 3 to 7, was found to be influenced by the changes in the relative concentration of the extract and of that of thiamine. The rate of inactivation of thiamine by the undialysable portion of the mustard extract was found to be directly proportional to the concentration of the vitamin.

Stability of the anti-thiamine factor of ragi.—The stability of the factor towards acid and alkaline pHs was tested. The extract was brought to pH 10 and left overnight. Another portion of the extract was kept overnight at pH 2.0. An extract at pH 5.6 served as a control. The different extracts were adjusted to pH 5.6 and tested for their activity towards thiamine

(25 μ g.). The extract was perfectly stable at pH 2 and pH 10, was shown by the results given in Table VII:—

TABLE VII.
Stability of ragi extracts at pH 2 and 10.

pH.	μ g. thiamine destroyed.
2.0	22.9
5.6	22.9
10.0	23.7

Inactivation of thiamine present in yeast and liver by the extracts of ragi, mustard seeds and cotton seed.—Ragi extract was prepared by autoclaving it with acetate buffer. The residue was removed by filtration and the filtrate was used as a source of the active factor. The C. W. extracts of mustard seed and cotton seed were employed for this experiment.

To 0.5 g. of yeast (dried brewer's) and 5 g. of ox liver, 10 ml. of extract of ragi, mustard or cotton seed respectively were added. The pH was adjusted to 5.6 by the addition of M/2 acetate buffer, and the flasks incubated overnight at 37°C. Two parallel incubations were carried out with yeast and liver alone and the same with the test extracts + 25 μ g. of thiamine. The amount of thiamine remaining in all the solutions was estimated. The relevant data are given in Table VIII:—

TABLE VIII.
Inactivation of thiamine in yeast and liver by the test extracts.

Materials.	μg. THIAMINE.		Percentage thiamine inactivated.
	Calculated.	Found.	
<i>Ragi extract.</i> —			
0.5 g. yeast	40.3	..
10 ml. ragi extract	0.0	..
0.5 g. yeast + 10 ml. ragi extract ..	40.3	0.0	100
0.5 g. yeast + 25 μg. thiamine ..	65.3	65.3	..
0.5 g. yeast + 25 μg. thiamine + 10 ml. ragi extract.	65.3	4.1	93.6
<i>Mustard seed extract.</i> —			
(1) 0.5 g. yeast	20.7	..
10 ml. mustard extract	0.0	..
0.5 g. yeast + 10 ml. mustard extract ..	20.7	4.35	78.7
0.5 g. yeast + 25 μg. thiamine ..	45.7	40.1	..
0.5 g. yeast + 25 μg. thiamine + 10 ml. mustard extract.	45.7	21.8	51.6

TABLE VIII—*concl'd.*

Materials.				μ g. THIAMINE.		Percentage thiamine inactivated.
				Calculated.	Found.	
(2)	5 g. liver	10.0	..
	5 g. liver + 10 ml. mustard extract	10	0.0	100.0
	5 g. liver + 25 μ g. thiamine	35.0	33.3	..
	5 g. liver + 25 μ g. thiamine + 10 ml. mustard extract.	35.0	21.1	40.0
<i>Cotton seed extract.—</i>						
	0.5 g. yeast	32.2	..
	10 ml. cotton seed extract	0	..
	0.5 g. yeast + 10 ml. cotton seed extract	30.2	25.5	15.6
	0.5 g. yeast + 25 μ g. thiamine	55.2	55.2	..
	0.5 g. yeast + 25 μ g. thiamine + 10 ml. cotton seed extract.	55.2	41.4	25.0

All the three extracts destroyed thiamine in yeast and liver, when they were mixed with the latter. With ragi extract complete destruction of thiamine in yeast was observed.

SUMMARY.

1. Certain foodstuffs, viz. ragi, rice polishings, green gram, mustard seed, cotton seed and linseed were found to contain a factor which rapidly inactivated thiamine *in vitro*.
2. This factor (anti-thiamine) was shown to be soluble in water but insoluble in salt solutions and was extracted with chloroform-water mixture.
3. It was found to be non-enzymic in nature.
4. On dialysis, the factor was resolved into two components one heat-labile and non-dialysable and the other heat-stable and dialysable. Either component was active towards thiamine.
5. Destruction of thiamine in materials rich in thiamine, viz. brewer's yeast, was observed when the factor was mixed with them.

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INACTIVATION OF THIAMINE BY CERTAIN FOODSTUFFS AND OIL SEEDS.

Part II.

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IN Part I of this paper (Bhagvat and Devi, 1944) (p. 131, this issue), it was shown that when thiamine was added to aqueous suspensions of certain cereals, pulses and oil seeds, it was rapidly inactivated as demonstrated by the absence of thiamine in the reaction mixture on testing by the thiochrome method. The inactivation was due to two factors—one thermolabile and non-dialysable and the other thermostable and dialysable. Both factors could be extracted with a chloroform-water mixture. The results obtained by the chemical method have since been confirmed by biological tests which provide a final criterion of thiamine activity. The test animals used for this purpose were (1) mosquito larvæ [*Aedes (stegomyia) albopictus*], (2) pigeon and (3) rat.

1. The technique used in the case of the mosquito larvæ was that described by Trager (1937). The eggs were sterilized by successive immersions in 80 per cent alcohol. They were collected on a small filter-paper and introduced aseptically into a tube containing sterile distilled water. As soon as the eggs hatched out, 5 larvæ were taken out by means of a sterilized dropper and introduced into each of the sterilized tubes containing the basal medium + the different test solutions. The tubes were incubated at 30°C. and the growth of the larvæ was observed daily till they reached the 4th instar. This usually took 8 to 10 days.

The liquid basal medium used consisted of 0.1 per cent glucose, 0.1 per cent salt mixture (Osborne and Mendel) and 0.2 per cent autoclaved brewer's yeast extract. It was supplemented with 50 µg. riboflavin and 50 µg. pantothenic acid. After adjusting the pH to 6.0, 5 ml. were pipetted into a series of test-tubes, a known aliquot of the test solutions was added and the volume was made up to 10 ml. The tubes were sterilized for 15 minutes at 15-lb. pressure. They were cooled to room temperature and 5 larvæ were introduced into each one of them. To two tubes after sterilization 2 µg. of thiamine were added. These served as control.

Preparation of the test solution.

(1) *Ragi extract*.—2.5 g. of powdered ragi (*Eleusine coracana*) was mixed with M/10 acetate buffer pH 5.6 and incubated overnight with 50 µg. of thiamine. The residue was removed on the centrifuge and washed repeatedly with distilled water. The centrifugate and the washings were mixed and made up to a known volume. An aliquot equivalent to half the solution, on chemical estimation was found to contain 0.2 µg. of thiamine instead of 25 µg. Another aliquot was dialysed for 72 hours against distilled water. The dialysate (RD) and the undialysable portion (RE) were preserved and portions equivalent to 2 µg. thiamine (on the basis of original thiamine content) of both fractions were used for tests with the larvæ.

(2) and (3) *Mustard seed and carp extracts*.—The extracts were prepared in the same way and dialysed for 72 hours. Both the dialysable and undialysable portions (= 2 µg. thiamine on the basis of original thiamine content) were used.

(4) *Sodium hydroxide-treated thiamine*.—A solution containing 100 µg. of thiamine was brought to pH 11 by the addition of 40 per cent NaOH and left overnight. It was brought

back to pH 4.0 and an aliquot on testing was found to contain only 0.8 μ g. thiamine instead of 25 μ g. One ml. of the solution containing 2 μ g. of thiamine (on the basis of original thiamine content) was used for the experiment.

(5) *Sulphite-treated thiamine*.—To a solution containing 100 μ g. of thiamine at pH 5.0 a small amount of sodium sulphite was added and the mixture kept standing overnight at room temperature (20°C. to 25°C.). The excess of sulphite was removed with baryta, excess of which was removed by the addition of H_2SO_4 . The solution was filtered and the filtrate was made up to 50 ml. An aliquot on testing was found to contain practically no thiamine. 0.5 ml. of the filtrate (= 2 μ g. thiamine on the basis of original thiamine content) was employed for the test.

The growth of the larvæ was observed for 10 days. The growth of the larvæ receiving 2 μ g. of pure thiamine was designated + + + + and the results are given in the Table :—

TABLE.
Growth response of mosquito larvæ.

Medium alone	+
.. + thiamine ¹	+ + + +
.. + thiamine ²	+
.. + RE	+ +
.. + .. + thiamine ¹	+ + + +
.. + RD	+ + + +
.. + ME	+ + +
.. + .. + thiamine ¹	+ + + +
.. + MD	+ + + +
.. + CE	+
.. + .. + thiamine ¹	+ + + +
.. + CD	+ + + +
.. + sulphite-treated thiamine	+
.. + NaOH-treated thiamine	+ +

Thiamine¹= thiamine added after sterilization of the tubes.

Thiamine²= " before " " " "

ME=non-dialysable portion of mustard seed extract. "

MD=dialysable " " " "

CE=non-dialysable " carp extract. " "

CD=dialysable " " " "

It will be seen from the Table that mosquito larvæ require thiamine for good growth. Growth in the medium without thiamine was very poor. They could not utilize such break-down products of thiamine as are obtained on autoclaving, or on treatment of the vitamin by sulphite or NaOH. However, they readily utilize, as judged by growth response, the break-down products formed from thiamine by the action of carp tissue. These were shown by Krampitz and Woolley (1944) to be 4-methyl-5-hydroxy-ethyl-thiazole and 2-methyl-4-amino-5-hydroxy-methyl-pyrimidine. The break-down products formed when thiamine was added to aqueous suspensions of ragi and mustard seed also stimulated growth. The nature of these products is not yet known ; attempts are being made to isolate them. The growth-promoting effect of the extracts could not be attributed to the presence of intact thiamine because (1) the two extracts were shown to be devoid of the vitamin by chemical tests and (2) even if small

amount of thiamine was present in the extracts, it would have been destroyed when the tubes were autoclaved. The thiamine break-down products obtained on autoclaving were unable to promote the growth of the larvæ.

2. *Test with pigeons (curative and preventive).*—Ragi extract was used for the tests with pigeons and rats.

Preparation of the extract.—Five grammes powdered ragi were mixed with 25 ml. of M/10 acetate buffer pH 5.6 and the suspension was autoclaved for 15 minutes at 15-lb. pressure. After cooling 1,000 μ g. of thiamine were added and the flask was incubated at 37°C. overnight with toluene as a preservative. The residue was removed on the centrifuge and washed repeatedly with distilled water. The washings and the centrifugate were mixed, made up to 100 ml. and filtered. A clear filtrate was obtained. One ml. of the filtrate which theoretically should contain 10 μ g. of thiamine, but was found to contain only 0.5 μ g., was given as a daily dose to pigeons.

Another extract was made in the same way for injections, but the concentrations of thiamine was so adjusted that 1 ml. theoretically should contain 50 μ g. of thiamine. - Actually it was found to contain only 6 μ g.

Adult pigeons were divided into three groups, the 1st group containing 6 and the 2nd and 3rd groups 12 birds each, and fed on a diet composed exclusively of washed raw milled rice, which was treated with sulphite to destroy traces of thiamine. The group 1, which served as a positive control, was given daily 1 ml. of a solution containing 10 μ g. of thiamine, the solution being poured into the crop through a pipette. This amount of thiamine was found to be sufficient to prevent polyneuritis. Pigeons in group 2 received daily 1 ml. of the ragi extract to which thiamine had been added as described above. Group 3 served as negative controls. The birds were weighed once a week.

The pigeons in group 1 (positive controls) lost weight during the first 2 to 3 weeks: after this period their weight remained more or less stationary. The negative controls showed a rapid fall in weight and after 3 to 4 weeks; some 67 per cent showed head retraction. The remainder died without showing head retraction, post-mortem examination revealing enlargement of the heart and pericarditis. The birds showing head retraction were given intravenously 0.5 ml. of ragi extract, which would have contained 25 μ g. of thiamine if no break-down of the vitamin had occurred. In preliminary experiments this quantity of thiamine was found adequate for the cure of head retraction when given by the intravenous route, leading to disappearance of retraction in 30 to 40 minutes and a cure lasting 5 to 6 days. When the ragi extract was injected, relief of head retraction was observed in 60 to 90 minutes. Head retraction recurred, however, within 40 hours. This seemed to be of the nature of a 'false cure', such as has been reported after the administration of glucose. The pigeons in the 2nd group receiving ragi extract showed a more gradual fall in weight than the negative controls, possibly due to traces of thiamine in the ragi extract, but 8 out of 12 ultimately developed head retraction. It was relieved by intravenous injection of 25 μ g. of thiamine, the cure lasting several days. These experiments suggest that thiamine added to an aqueous suspension of ragi is rendered biologically inactive for pigeons.

3. *Tests (curative and preventive) with rats.*—The ragi extract used in these experiments was the same as in the pigeon tests, except that it was diluted with an equal volume of water so that 1 ml. = 5 μ g. thiamine. This is designated treated ragi extract. Ragi extract without thiamine is referred to as untreated ragi extract.

Young rats weighing 45 g. to 65 g. were caged individually over a wide mesh-screening and were fed *ad libitum* on a basal diet consisting of sucrose 58 g., purified thiamine-free casein 18 g., salt mixture (McCollum and Davis) 4.0 g., gingelly oil 10 g., and autoclaved brewer's yeast 10 g.; one drop of shark-liver oil was given per rat per day. The diet was mixed and given in small trays.

The experiments were repeated with two separate batches of animals. In the 1st batch sucrose as obtained from the market was used. This was found to contain thiamine, resulting in the prolongation of the time of onset of thiamine deficiency. In the 2nd batch sucrose purified by repeated washings with 95 per cent ethyl alcohol was employed and the time taken for the onset of the deficiency was substantially reduced.

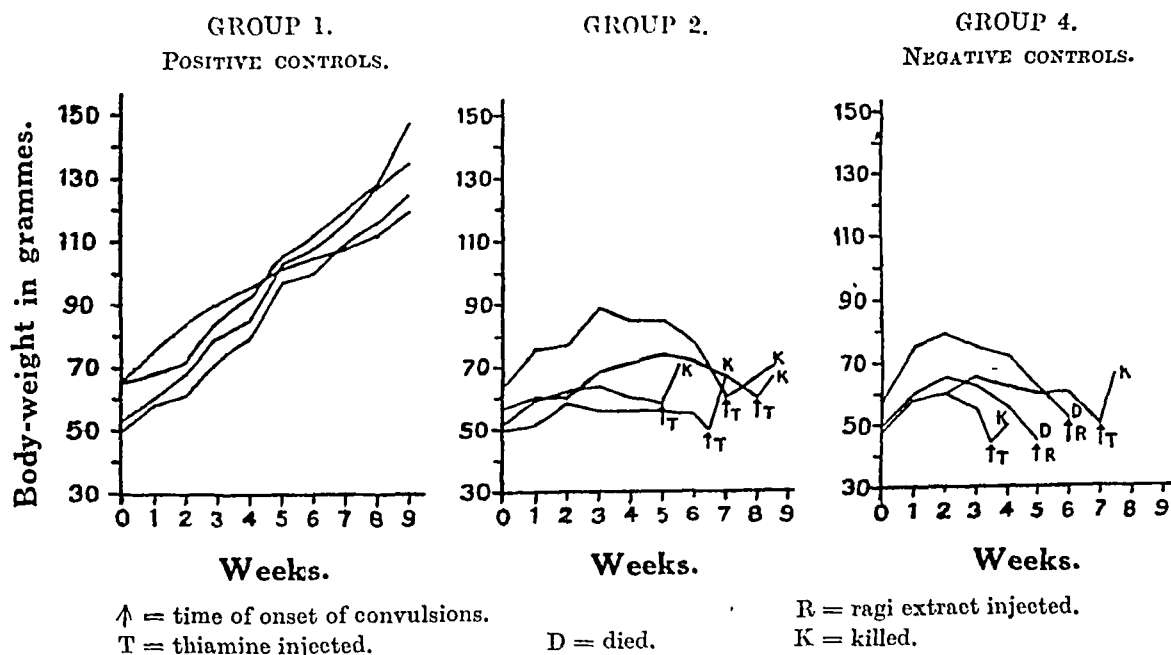
The rats were distributed into the following groups :—

Group.	Number of rats in each group.		μ g. thiamine fed daily per rat.	ml. ragi-treated extract per rat per day.	ml. untreated ragi extract per rat per day.
	I	II			
I. Positive control (receiving thiamine) ..	6	5	5.0
II. Receiving treated ragi extract ..	12	7	..	1	..
III. Receiving untreated ragi extract ..	5	5	5.0	..	1
IV. Negative control (no thiamine) ..	12	8

The vitamin solution, treated and untreated ragi extracts were given in small trays with a pinch of sugar. After the rats had licked up the solution, the diet trays were put inside the cages. In the 3rd group, the rats were fed by hand 5 μ g. thiamine by means of a dropper and after a lapse of an hour, trays containing untreated ragi extracts were put inside the cages. This precaution is necessary in order to prevent the inactivation of thiamine in the stomach by ragi extract.

The rats were weighed weekly. Typical results are represented in the Graph :—

GRAPH.



Weight curves of rats receiving basal diet supplemented by 5 μ g. thiamine and treated ragi extract respectively.

The positive controls grew steadily thereby indicating that 5 μ g. of thiamine per rat per day is adequate for growth. The negative controls, after 2 to 3 weeks, on the basal diet declined in weight, lost appetite and passed into characteristic convulsions. The sick rats were then divided into two batches. The 1st batch was given subcutaneously 50 μ g. of pure thiamine and

the 2nd batch 1 ml. of treated ragi extract (to which 50 μ g. thiamine were added). The rats in the 1st batch recovered within 2 hours and were destroyed 2 to 3 days after recovery when they showed an increase in weight. The rats in the 2nd batch did not recover and death occurred in 2 to 3 hours. This demonstrates the inactivation of thiamine on its addition to ragi extract. The rats in the 3rd group did not grow properly at the beginning of the experiment and this was attributed to the intermixing of ragi extract with the vitamin in the gastrointestinal tract, thereby probably inactivating it. This could, however, be prevented by feeding the vitamin nearly a hour before the extract was given. Some of the rats then showed gain in weight, which was maintained over the whole experimental period. The animals in the 2nd group behaved in the same way as the negative controls with the difference that the onset of polyneuritic manifestations, convulsions, loss of appetite, difficulty in the use of the limbs, etc., was delayed by a few days possibly due to traces of thiamine in the ragi extract. The above signs were relieved on the administration of 50 μ g. thiamine, and the rats gained in weight. From 74 to 86 per cent of the rats in group 2 showed typical polyneuritic symptoms. The rat experiments appeared to demonstrate conclusively that thiamine is rendered biologically inactive for rats by treatment with ragi extract.

DISCUSSION.

In Part I of this paper (Bhagvat and Devi, *loc. cit.*), evidence has been brought forward to show the presence of a factor in certain foods, viz. ragi, rice polishings, wheat germ, green gram, mustard seeds, cotton seed and linseed, which rapidly inactivated thiamine *in vitro*. The factor was extracted by chloroform-water mixture and was found to be non-enzymic in nature. Free as well as combined thiamine was inactivated by the factor, when the latter was added to thiamine-rich materials such as brewer's yeast. That thiamine is rendered biologically inactive when added to ragi extract has also been shown by biological experiments with rats and pigeons. Development of polyneuritis in pigeons fed exclusively on rice bran was observed by Williams (1929), while Hart, Miller and McCollum (1916) and Rommel and Vedder (1927) produced in pigs a condition closely resembling thiamine deficiency by feeding them on diets consisting of wheat products and cotton seed respectively. A toxic substance present in the outer coats of rice, etc., which appears to destroy thiamine *in vivo*, was suggested as the causative factor. Rice polishings, wheat germ, etc., have a high thiamine content and the suggested presence of a thiamine-inactivating factor is somewhat remarkable. The presence of a vitamin and anti-vitamin factor in one and the same food is observed in the hen's egg. The yolk is rich in biotin, while the egg white is rich in avidin—the 'anti-biotin factor'.

The observations presented in Parts I and II of this series may affect accepted beliefs regarding thiamine requirements and utilization in man. The foods which were found to contain the thiamine-inactivating factor are commonly used in India. The factor was found to be heat-stable and was shown to destroy thiamine *in vitro* when mixed with thiamine-rich foods. The action of anti-thiamine factors *in vivo* requires further study.

SUMMARY.

1. The destruction of thiamine by ragi extract was confirmed by biological experiments with (a) larvæ of the mosquito—*Aedes albopictus*, (b) rats and (c) pigeons.

2. Mosquito larvæ were found to utilize the break-down products of thiamine produced by the action of ragi extract, though they were unable to utilize break-down products of thiamine obtained by autoclaving, or by sulphite or NaOH treatment of the vitamin.

3. Rats and pigeons when fed with ragi extract to which thiamine was added in amounts adequate for their growth, developed signs characteristic of thiamine deficiency which were relieved by administration of thiamine.

We are grateful to Mr. B. N. Mohan of the Malaria Institute, Coonoor, for the supply of sterilized eggs of the mosquito—*Aedes (stegomyia) albopictus*.

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LIBERATION OF TYROSINE, TRYPTOPHANE, CYSTINE AND ARGININE FROM PROTEINS.

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For the complete liberation of tyrosine, tryptophane, cystine and arginine from proteins, hydrolysis for 18 to 30 hours is usually recommended. Folin and Merenzi (1929), van Slyke (1911, 1912), and Henriques and Gjaldback (1910) found, however, that casein and peptone could be completely hydrolysed by autoclaving the solutions in 3NHCl at 150°C. for 90 minutes. Similar results were obtained by Borches and Berg (1942) with zein on autoclaving it with 33 per cent H_2SO_4 for one hour. More recently, Leiben (1943) has shown that the hydrolysis of proteins and peptones at high temperatures could be accelerated by certain catalysts, thereby considerably shortening the time required for complete hydrolysis.

In this paper, experiments are reported with different proteins to show that the above-mentioned essential amino acids can be completely released on autoclaving the protein solutions for 30 to 45 minutes at 15-lb. pressure. For comparison, results obtained by other methods of hydrolysis are incorporated.

EXPERIMENTAL.

Seven different proteins from a variety of sources were taken for experiment. Gelatin and casein bought on the market were used. The other proteins were prepared according to methods given below. All analytical values were expressed as the percentage of total nitrogen on an ash- and moisture-free basis.

Egg albumin was prepared according to the method of Hopkins and Pinkus (1898), with the difference that the precipitated albumin was denatured by heating, washed with water till free from ammonium sulphate. It was finally washed with alcohol, acetone and ether and dried *in vacuo* over sulphuric acid. The total globulins of green gram (*Phaseolus radiatus*) were prepared by the dilution method of Niyogi, Narayana and Desai (1932), and wheat gliadin by the alcohol extraction method as described for the extraction of eleusin from ragi (*Eleusine coracana*) by Narayana and Norris (1928). Wheat gluten was obtained by washing a dough made out of wheat flour repeatedly with water until all the starch was washed away. Gluten, which remained behind, was finally washed with alcohol, acetone and ether and dried *in vacuo* over sulphuric acid.

Tapioca protein used in this investigation was prepared as follows : fresh tapioca (*Manihot utilissima*) roots were cleaned and crushed in a stone mortar with a small amount of water. The juice was pressed out and starch removed on the centrifuge. The solution was adjusted to pH 5.0 and heated to boiling. The coagulated proteins were separated by centrifugation, washed repeatedly with water and finally with alcohol, acetone and ether. They were then dried *in vacuo* over sulphuric acid. A dark-coloured protein was obtained.

Tyrosine, tryptophane and cystine were determined directly by the method of Folin and Merenzi (*loc. cit.*) with the difference that fuller's earth (0.1 g. per 25 ml. of protein hydrolysate) was used instead of kaolin. Preliminary trials showed no adsorption of the three amino acids

on fuller's earth. A portion of the hydrolysate used in the estimation of cystine was employed for the colorimetric estimation of arginine according to the method of Weber (1930). It was observed that fuller's earth did not remove substances which interfere with the arginine estimation. These interfering substances, however, were removed by adsorption on norit, without loss of arginine. The amount of norit used per 25 ml. of the hydrolysate was 0.1 g. The tyrosine, tryptophane, cystine and arginine contents of the different proteins are given in Table I. The proteins were hydrolysed in a boiling water-bath for 18 to 20 hours in accordance with the usual method referred to above.

TABLE I.

Tyrosine, tryptophane, cystine and arginine content of various proteins.

Protein.			Tyrosine.	Tryptophane.	Cystine.	Arginine.
Egg albumin	2.50	0.93	1.33	4.91
Casein	2.53	0.5
Total globulins of green gram	2.29	0.58	0.8	6.32
Wheat gluten	2.49	0.53	1.60	3.58
„ gliadin	1.86	0.60	1.87	2.56
Tapioca protein	1.67	1.08	1.26	16.51
Gelatin	18.73

Liberation of tyrosine, tryptophane, cystine and arginine by autoclaving.—The rate of liberation of the different amino acids on autoclaving was first studied. Casein was used for the liberation of tyrosine and tryptophane, egg albumin for that of cystine, and gelatin for that of arginine. These three proteins were chosen because they are rich in the particular amino acid under test. Portions of 0.1 g. of each of the proteins were weighed out and put into test tubes, which were divided into 3 series. To one (containing casein), 2 ml. of 20 per cent NaOH were added, and to the others (containing egg albumin and gelatin respectively) 2 ml. of 10 per cent H₂SO₄. The tubes were autoclaved at 15-lb. pressure and 120°C. The tubes were cooled and those containing alkali were neutralized. The solutions were made up to 25 ml. and treated with 0.1 g. of fuller's earth. An aliquot of the acid hydrolysate was treated with norit and the filtrate used for the estimation of arginine. Tyrosine, tryptophane, cystine and arginine were estimated. Results are given in Table II :—

TABLE II.

Rate of liberation of tyrosine, tryptophane, cystine and arginine.

Time of autoclaving in minutes.	CASEIN.		EGG ALBUMIN.		GELATIN.
	Tyrosine.	Tryptophane.	Cystine.	Arginine.	
15	2.16	0.34	0.95	14.04	
30	2.53	0.50	1.12	15.16	
45	2.40	0.42	1.27	18.03	
60	2.12	0.39	1.04	17.23	

It will be seen from Table II that tyrosine and tryptophane seem to be completely liberated from casein on autoclaving for 30 minutes, while the complete liberation of cystine from egg albumin and arginine from gelatin required 45 minutes. If the autoclaving was prolonged, loss of the amino acids occurred.

In order to test the reliability of the method, recovery experiments with different amino acids were carried out. One milligram of cystine was added to egg albumin and a similar amount of arginine to gelatin. After the addition of 10N H_2SO_4 , the tubes were autoclaved for 45 minutes at 15-lb. pressure and cystine and arginine estimated (Table III) :—

TABLE III.

Recovery of amino acids added to proteins.

		AMINO ACID.		Percentage recovery of added amino acid.
		Added, mg.	Recovered, mg.	
Cystine	..	1.0	0.934	93.4
Arginine	..	1.0	0.925	92.5

The recovery of cystine and arginine added to egg albumin and gelatin respectively was almost complete.

The amino acid make-up of the proteins was then investigated. The proteins were subjected to autoclaving for 30 minutes for the liberation of tyrosine and tryptophane and for 45 minutes for the liberation of cystine and arginine. Table IV gives the results :—

TABLE IV.

Liberation of tyrosine, tryptophane, cystine and arginine from proteins by autoclaving.

Protein.			Tyrosine.	Tryptophane.	Cystine.	Arginine.
Egg albumin	2.55	0.92	1.27	4.95
Casein	2.54	0.51
Total globulins of green gram	2.22	0.57	0.81	6.15
Wheat gluten	2.46	0.53	1.54	3.50
„ gliadin	1.81	0.60	1.95	2.63
Tapioca protein	1.63	1.07	1.29	17.03
Gelatin	18.03

The values recorded in Table IV agree well with those given in Table I, thereby indicating the suitability of autoclaving as a means of hydrolysing the proteins in question. This procedure involves a considerable economy of time and research material and yields results which are in agreement with those obtained by other methods. Another interesting point brought out by the results is the adequacy of tapioca protein as regards the essential amino acids,

tyrosine, tryptophane, cystine and arginine. It is especially rich in arginine. The low nutritive value of tapioca root thus appears to be attributable to the quantity rather than the quality of the protein it contains.

SUMMARY.

1. Seven-proteins prepared from a variety of sources, after 18 to 20 hours hydrolysis (as recommended in the older methods), were analysed for 4 essential amino acids, viz. tyrosine, tryptophane, cystine and arginine.

2. Complete liberation of the above amino acids was achieved by hydrolysis of the proteins in the autoclave for 30 to 45 minutes at 15-lb. pressure and at 120°C.

3. The results given by the new procedure agreed well with those given by earlier methods.

4. Recovery of cystine and arginine added to protein solutions prior to autoclaving was good.

5. Tapioca protein was found to contain the essential amino acids in question in adequate amounts.

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EGG-WHITE INJURY (INDUCED BIOTIN DEFICIENCY) IN RICE-MOTH LARVÆ (*CORCYRA* *CEPHALONICA* ST.).

BY

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BOAS (1927) discovered that an unusual type of injury was produced in rats by feeding them large quantities of raw egg-white. The rats showed eczema-like dermatitis and loss of hair, which could be cured by a factor present in potato, milk, yeast, etc., which was named, 'protective factor X'. This factor has been extensively studied by György (1939), who was able to isolate it from liver and called it 'vitamin H'. He showed later (György, 1940) that vitamin H was similar in its properties to the biotin isolated by Kögl from egg-yolk.

Egg-white loses its property of causing dermatitis and alopecia in rats when it is heated. Eakin, Snell and Williams (1941) purified the heat-labile factor, termed it 'avidin' and showed that it combines with biotin, thereby preventing the latter from becoming available to the organism. The amount of avidin in uncooked egg-white is more than enough to neutralize the amount of biotin in egg-yolk, though after cooking the avidin is destroyed. On a diet containing a large amount of raw egg-white, chicks like rats develop a typical dermatitis, which is cured by biotin (Hegsted, Mills, Briggs, Elvehjem and Hart, 1942). Sydenstricker, Singal, Briggs, de Vaughn and Isbell (1942) have recently described the effect of biotin deficiency in human beings who were consuming large quantities of raw egg-white.

There is evidence to show that biotin is necessary for certain lower organisms, such as *Lactobacillus casei* (Shull, Hutchings and Peterson, 1942), and *Clostridium butylicum* (Lampen, Bahler and Peterson, 1942), and methods of assay for biotin have been developed, based on the growth of these bacteria.

Biotin has also been shown to be necessary for the growth of the flour beetle, *Tribolium confusum*, and also to be required by certain other classes of insects such as *Lucilia* and *Drosophila* (Fraenkel and Blewett, 1942; Rosenthal and Reichstein, 1942). These studies were carried out using synthetic diets which did not contain biotin, and it was found that the growth of the insect took place only after the addition of biotin to the diet.

In view of the above findings, it was decided to investigate whether rice-moth larvæ (*Corcyra cephalonica* St.) require biotin for their growth. It was found that on synthetic diets containing raw egg-white or avidin concentrates the larvæ grew very little and died in about 4 weeks from egg-white injury, which, it is clear, is the same as induced biotin deficiency. This effect was not seen, if, at an early stage, the larvæ were transferred to diets containing biotin or biotin concentrates in sufficient quantities.

EXPERIMENTAL.

The preparation of a diet complete in necessary vitamins, on which the growth of rice-moth larvæ was as good as on whole wheat, has already been described by Sarma (1943). Briefly, it is as follows: whole-wheat flour was extracted with 3 per cent sodium-chloride solution for 3 days, coupled with autolysis, to remove the water-soluble vitamins. Sugar and salt mixture of McCollum and Davis were added at a 4 per cent level in each case. Thiamine, riboflavin, nicotinic acid, pyridoxine and calcium pantothenate were added in the proportion of 10 µg., 5 µg., 50 µg., 5 µg., and 15 µg. per g. of diet respectively. To 5 g. of this diet, 4 ml. of raw egg-white were added and thoroughly mixed. To another portion of 5 g. of diet, 4 ml. of egg-white heated on a boiling water-bath for 5 minutes were added and the diet mixed.

Rice-moth larvæ, which had been fed on the whole-wheat diet for 10 to 12 days after hatching, were put on the various diets in petri dishes. They were kept in the incubator at 30°C. and their growth studied by taking their weights every week. Table I gives the weights of larvæ fed on different diets.

TABLE I.
Weight of larvæ on diets containing raw and cooked egg-white.
Weight in mg. of ten larvæ.

Diet.	Initial weight.	Weight after 7 days.	Weight after 14 days.	Weight after 21 days.	After 28 days.
1. 5 g. B.D. + all vitamins + 4 ml. of raw egg-white.	4.80	30.10	35.60	29.60	All dead.
2. 5 g. B.D. + all vitamins + 4 ml. of cooked egg-white.	4.64	51.60	138.60	208.60	All pupated.
3. B.D. + all vitamins	4.55	49.78	125.60	200.70	„
4. Whole-wheat diet	4.60	50.60	129.60	210.90	„

From Table I. it is clear that there is present in raw egg-white, a toxic factor which prevents the growth of rice-moth larvæ and causes death in about 28 days. On heating the egg-white, however, the toxic factor is destroyed and the larval growth on such a diet is as good as on whole wheat or on the basal diet + all vitamins.

Parsons and Kelly (1933) have shown that the capacity of dietary egg-white to produce dermatitis in the rat is destroyed by peptic digestion or by mild treatment with hydrochloric acid. But that this effect was not due merely to denaturation was shown by the persistence of the toxicity in egg-white denatured by strong alcohol. The following experiments were carried out to discover whether the toxic factor which affects the growth of larvæ has the same characteristics as that of the factor described by Parsons and Kelly.

Raw egg-white was incubated at 37°C. for 24 hours with dilute hydrochloric acid of N/2 strength. A second aliquot of raw egg-white was incubated with pepsin in acid medium, while a third was run into absolute alcohol to make a final concentration of 75 per cent. These differently treated egg-white preparations were mixed with 5 g. portions of the complete diet (i.e. basal diet + all vitamins), in the same proportions as detailed above. Larvæ fed on whole wheat for 10 days were placed on these different diets and their growth studied. Table II gives the results :—

TABLE II.
The effect of treating egg-white by various ways on the growth of larvæ.
Weight in mg. of ten larvæ.

Diet.	Initial weight.	Weight after 7 days.	Weight after 14 days.	Weight after 21 days.	After 28 days.
1. B.D. + all vitamins + HCl treated raw egg-white.	4.20	40.43	108.80	189.40	All pupated.
2. B.D. + all vitamins + pepsin digested raw egg-white.	4.15	39.13	105.60	185.60	„
3. B.D. + all vitamins + egg-white denatured by strong alcohol.	4.50	23.06	26.95	22.75	All dead.

TABLE II—*concl'd.*

Diet.	Initial weight.	Weight after 7 days.	Weight after 14 days.	Weight after 21 days.	After 28 days.
4. B.D. + all vitamins + egg-white cooked for 5 minutes in a boiling water-bath.	4.34	47.11	130.60	203.40	All pupated.
5. B.D. + all vitamins + raw egg-white.	4.44	28.03	32.10	24.00	All dead.
6. B.D. + all vitamins	4.36	41.81	123.80	200.60	All pupated.

From the results in Table II, it is clear that the toxic factor affecting the rice-moth larvæ possesses the same properties as the toxic factor present in egg-white, which affects rats, chicks, etc. The latter factor is now known as the anti-biotin factor or avidin and has been prepared in a concentrated form from raw egg-white. The next step was to prepare an avidin concentrate from raw egg-white, mix it with the diet, and study larval growth on such diets.

Avidin concentrate was prepared according to the method of Eakin, Snell and Williams (*loc. cit.*). One hundred ml. of fresh egg-white were added to 400 ml. of acetone and the coagulum was washed with 50 ml. of water. It was then thoroughly suspended and washed with 200 ml. of 1 per cent sodium-chloride solution and the filtrate retained. The filtrate was five-sixths saturated with ammonium sulphate and filtered, the precipitate being discarded. The active fraction was then salted out by complete saturation with ammonium sulphate. After filtration, the material was dissolved in a small volume of water and dialysed in a cellophane bag against tap-water. The total volume of avidin concentrate obtained was 50 ml. Diets were prepared incorporating 2 ml. and 4 ml. of avidin concentrates in 5 g. portions of the complete diet. Two ml. of avidin concentrate were steam-sterilized for 30 minutes to destroy the anti-biotin factor and then mixed with 5 g. of diet. Larvæ, 10 days old, were taken from the whole-wheat diet and put on the above diets. Their weight increments are given in Table III:—

TABLE III.

The effect of avidin concentrate on the larvæ.

Weight in mg. of ten larvæ.

Diet.	Initial weight.	Weight after 7 days.	Weight after 14 days.	Weight after 21 days.	After 28 days.
1. 5 g. B.D. + all vitamins + 4 ml. of raw egg-white.	4.00	22.10	34.44	29.00	All dead.
2. 5 g. B.D. + all vitamins + 2 ml./50 ml. of avidin concentrate.	3.95	28.1	72.80	61.62	"
3. 5 g. B.D. + all vitamins + 4 ml./50 ml. of avidin concentrate.	4.16	22.70	64.10	30.75	"
4. 5 g. B.D. + all vitamins + 4 ml./50 ml. of avidin concentrate steam-sterilized for 30 minutes.	4.27	36.72	118.50	195.60	All pupated.
5. B.D. + all vitamins	4.30	41.25	125.60	215.40	"

The results indicate that avidin concentrates are as toxic as raw egg-white to the rice-moth larvæ. Though the larvæ grew a little in the initial stages, they died in 28 days.

Rice-moth larvæ, which had been fed on the whole-wheat diet for 10 to 12 days after hatching, were put on the various diets in petri dishes. They were kept in the incubator at 30°C. and their growth studied by taking their weights every week. Table I gives the weights of larvæ fed on different diets.

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3. B.D. + all vitamins	4.55	49.78	125.60	200.70	„
4. Whole-wheat diet	4.60	50.60	129.60	210.90	„

From Table I, it is clear that there is present in raw egg-white, a toxic factor which prevents the growth of rice-moth larvæ and causes death in about 28 days. On heating the egg-white, however, the toxic factor is destroyed and the larval growth on such a diet is as good as on whole wheat or on the basal diet + all vitamins.

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Raw egg-white was incubated at 37°C. for 24 hours with dilute hydrochloric acid of N/2 strength. A second aliquot of raw egg-white was incubated with pepsin in acid medium, while a third was run into absolute alcohol to make a final concentration of 75 per cent. These differently treated egg-white preparations were mixed with 5 g. portions of the complete diet (i.e. basal diet + all vitamins), in the same proportions as detailed above. Larvæ fed on whole wheat for 10 days were placed on these different diets and their growth studied. Table II gives the results :—

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2. B.D. + all vitamins + pepsin digested raw egg-white.	4.15	39.13	105.60	185.60	„
3. B.D. + all vitamins + egg-white denatured by strong alcohol.	4.50	23.06	26.95	22.75	All dead.

SUMMARY.

1. Egg-white injury can be produced in rice-moth larvæ by feeding them on a diet containing raw egg-white. The larvæ fed on such a diet die off in about 4 weeks.
2. The toxic factor in raw egg-white is destroyed by mild acid hydrolysis, and by peptic digestion. The toxicity is not lost on denaturation with strong alcohol.
3. The toxic factor, which is present in the protein fraction, is present in an avidin concentrate prepared from raw egg-white.
4. The growth response of biotin-deficient larvæ appears to be proportional to the amount of biotin present in the diet.

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STUDIES ON THE DESTRUCTION OF VITAMIN A IN SHARK-LIVER OIL.

Part I.

DESTRUCTION DURING NORMAL STORAGE : STANDARDIZATION OF PULFRICH PHOTOMETER FOR THE ESTIMATION OF VITAMIN A.

BY

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WIDE variations in the stability of vitamin A under different conditions have been reported (Wokes and Willimott, 1927; Drummond and Hilditch, 1930; Marcus, 1931; MacWalter, 1934; Smith, 1939). When properly stored cod-liver oil has been found to retain its vitamin A content for long periods (Jones, 1928; Evers, 1929; Ewe, 1933). However, Iyengar and Mukerjee (1939) observed that 69 per cent of the samples of imported cod-liver oil examined by them were below the specifications of the British Pharmacopœia (1932), and suggested that the conditions of storage in the tropical climate may play an important part in the maintenance of the quality of cod-liver oils. In this laboratory Ramasarma, and Ramaswamy and Banerjee (unpublished results) have observed that the vitamin A in shark-liver oil prepared in India was destroyed rapidly on keeping. Experiments carried out by the author on the extent of destruction over long periods of storage under normal conditions are reported in this paper.

Three methods are available for the estimation of vitamin A : (i) the biological, (ii) the physical (measuring the absorption of ultra-violet light at 3280 Å on the quartz spectrophotometer or the simplified Hilger vitameter A), and (iii) the chemical (measuring the intensity of the blue colour resulting from reaction with antimony trichloride in the Lovibond tintometer). With pure vitamin A the blue colour of the Carr-Price reaction exhibits an absorption maximum at 6100–6200 Å, and the determination of the extinction coefficient at that wave-length has been adopted by many workers as a more accurate method for the estimation. Jensen and With (1939) and Fujita and Sakamoto (1941) have determined the intensity on the Pulfrich photometer using the filter S.61. This instrument has been standardized for the estimation of vitamin A in Indian shark-liver oils.

EXPERIMENTAL.

A number of samples of shark-liver oil were bought from different localities and their vitamin A contents determined within a week after their receipt. The oils were stored at room temperature in the dark and in the same bottles in which they were supplied. At intervals, the vitamin A was estimated. The determinations were made on the unsaponifiable matter in alcohol on the Hilger vitameter A. Some of the results are given in Table I.

Commercial shark-liver oils A, B, C and D were diluted with ground-nut oil by the suppliers.

TABLE I.

Destruction of vitamin A during storage of shark-liver oil.

Sample of oil.					Period of storage.	Vitamin A E $\frac{1\%}{1 \text{ cm.}} \times 1000.$	Percentage loss in vitamin A.
Pure shark-liver oil	{	0	4,480	..
					0 weeks	3,400	24
					23 months	1,860	58
Commercial shark-liver oil A	{	0	700	..
					4 months	530	24
					7 ..	480	31
Commercial shark-liver oil B	{	0	1,180	..
					8 weeks	910	23
Commercial shark-liver oil C	{	0	1,430	..
					18 months	860	40
Commercial shark-liver oil D	{	0	760	..
					18 months	380	50
Upjohn's cod-liver oil	{	0	1,360	..
					11 months	1,310	4

ESTIMATION OF VITAMIN A ON THE PULFRICH PHOTOMETER.

A solution of the shark-liver oil in chloroform (0.3 c.c.) was placed in a 1 cm. cell, a drop of acetic anhydride was added and 3 c.c. of a solution of antimony trichloride (saturated at 20°C.) in purified chloroform was quickly dropped in from a microburette. The extinction value was taken with filter S.61 as soon as possible after the addition (within 3 seconds) by rotating the graduated drum, so that the two halves of the field of view were uniformly illuminated. Three to 5 readings were taken, the cells interchanged and corresponding readings taken on the other side. The mean value was given as the result.

Tests with three samples of oil showed that Beer's law was obeyed fairly closely below an extinction value of 0.5; above that the E value was not directly proportional to the concentration of the oil in the solution (Table II). But, with the unsaponifiable fraction of the oil, Beer's law was obeyed at much higher concentrations also.

TABLE II.

Colour reaction and Beer's law.

Sample of oil.		Weight of oil in cell, mg.	E Value.	E \times 10 Weight of oil.	E value. (via unsap.).	E \times 10 Weight of oil.
A	..	0.75	0.115	1.53	0.15	2.0
		1.50	0.22	1.47
		3.0	0.43	1.43	0.60	2.0
		4.5	0.575	1.28
		9.0	0.815	0.91	1.7	1.9

TABLE II—*concl'd.*

Sample of oil.	Weight of oil in cell, mg.	$\frac{E}{\text{Value.}}$	$\frac{E \times 10}{\text{Weight of oil.}}$	$\frac{E \text{ value.}}{(\text{via unsap.})}$	$\frac{E \times 10}{\text{Weight of oil.}}$
B	0.75	0.135	1.7	0.18	2.4
	1.50	0.26	1.73
	3.0	0.51	1.7	0.71	2.37
	4.5	0.615	1.37	1.1	2.47
	9.0	0.89	0.99
	30.0	1.21	0.04
C	0.2	0.24	12.0
	0.25	0.29	11.6
	0.5	0.605	12.1
	1.0	1.05	10.5

Measurements in routine analysis were made on the whole oil, between extinction values 0.3 and 0.5. The extinction coefficient has been calculated as suggested by Jensen and With (*loc. cit.*) :—

$B_{1 \text{ cm.}}^{1\%} S.61 = \frac{E_n}{s \times c}$ where E_n denotes the mean value of n experiments, s the thickness of the cell, and c the concentration expressed in g. per 100 c.c. of the solution of oil in the cell (original solution plus reagent) and therefore $1/11$ of the concentration of the original solution.

The relation between $B_{1 \text{ cm.}}^{1\%}$ and $E_{1 \text{ cm.}}^{1\%}$ at 3280 \AA was determined for 10 samples of oil (Table III) :—

TABLE III.
Determination of the conversion factor.

Sample of oil.	$B_{1 \text{ cm.}}^{1\%}$	$E_{1 \text{ cm.}}^{1\%}$ Vitameter.	$\frac{B_{1 \text{ cm.}}^{1\%}}{E_{1 \text{ cm.}}^{1\%}}$	Vitamin A in I.U. per g. $\frac{E_{1 \text{ cm.}}^{1\%}}{E_{1 \text{ cm.}}^{1\%}} \times 1600$	$\frac{\text{I.U. per g.}}{B_{1 \text{ cm.}}^{1\%}}$
1	34.3	20.40	1.68	32.640	950
2	33.3	16.6	2.01	26.560	800
3	23.1	13.6	1.70	21.760	940
4	13.3	7.4	1.80	11.810	890
5	7.59	6.8	1.12	10.890	1,430
6	4.73	3.6	1.31	5.760	1,220
6	4.73	*2.8	1.69	4,480	950
7	5.28	3.3	1.60	5,280	1,000
7	5.28	*3.0	1.76	4,800	910
8	3.46	1.74	1.99	2,780	800
9	1.23	0.38	3.24	610	500
10	0.76	0.28	2.71	450	590
AVERAGE	1.92	..	910

* Determined on the unsaponifiable matter.

The extinction coefficient at 3280 \AA was measured on the whole oil in petrol solution on the Hilger vitameter A. Vitamin A potency in terms of international units per gramme of oil was calculated using the conversion factor 1600. On this basis, the factor for converting $B_{1 \text{ cm.}}^{1\%}$ into international units for shark-liver oil worked out to be 910 (average).

The extinction coefficients determined on the Hilger vitameter A agreed very well with those measured at 3280 \AA using the Hilger quartz spectrograph (E.316) fitted with the Spekker photometer (Table IV):—

TABLE IV.

Accuracy of the vitameter A and irrelevant ultra-violet absorption by the oil.

Sample of oil.	Whole oil	UNSATURIFIABLE FRACTION.	
	$E_{1 \text{ cm.}}^{1\%}$ vitameter.	$E_{1 \text{ cm.}}^{1\%}$ vitameter.	$E_{1 \text{ cm.}}^{1\%}$ spectrograph.
6	3.6	2.80	2.85
7	3.3	3.07	3.00
4	7.40	7.10	7.20

The extinction value on the vitameter A was noted at three different concentrations of the oil, at least five readings being taken at each concentration. The mean value for $E_{1 \text{ cm.}}^{1\%}$ was given as the result.

An appreciable proportion of the absorption at 3280 \AA in the whole oil is seen to be due to factors other than vitamin A which can be removed by saponification. The unsaponifiable matter was isolated by Rajagopal's (1941) modification of the method of Edisbury and Morton (1935). Absolute alcohol was used as the solvent for the estimation.

DISCUSSION.

The vitamin A in commercial samples of shark-liver oil was destroyed rapidly on keeping in the dark at room temperature (20°C. to 30°C.). Sundararajan (private communication) at the Nutrition Research Laboratories, Coonoor, observed an average loss of over 53 per cent in 11 samples of undiluted shark-liver oil kept at 38°C. in an incubator for 18 months. One of the reasons for the comparatively lower vitamin A potency of shark-liver oil bought in the market is possibly the destruction of the vitamin during the period of storage before reaching the consumer. In the interests of the future of the industry in India, a systematic study of the methods of manufacture and preservation of shark-liver oil is necessary to find out the causes of this deterioration in order to prevent it.

It has been found that the blue colour produced by the reaction of vitamin A with antimony trichloride can be measured more easily and accurately on the Pulfrich photometer using the filter S.61 than on the Lovibond tintometer. Even with considerable experience it is difficult to get a perfect match on the latter instrument. The interference of the red component, particularly in rancid oils, was eliminated by the filter.

The ratio of the extinction coefficient of the Carr-Price reaction at its absorption maximum (S.61) and the extinction coefficient at 3280 \AA is of great importance in determining the utility of the reaction as a quantitative method for the assay of vitamin A. Jensen and With (*loc.*

cit.), working with livers of different species of animals, obtained a ratio less than that reported in this paper. The variation in this ratio (Table III) is wide which indicates that the Carr-Price reaction when carried out on the whole oil is of limited utility.

The factor for converting $B_{1\text{ cm.}}^1\%$ for shark-liver oil into international units varied from 500 to 1430 in 10 samples, with an average of 910. With (1941) reported that for various substances tested, the factor ranged from 500 to 2000. The higher conversion factors noted for samples 5 and 6 were due to the irrelevant absorption by the glycerides at 3280 Å, since on removing the interference by saponification, the values fell near the average. By determining the $B_{1\text{ cm.}}^1\%$ value also on the unsaponifiable matter, possibly, the factors can be obtained within a narrower range, since Rajagopal (*loc. cit.*) and Seshan (1940) have shown that the development of maximum colour is inhibited in the whole oil. Edisbury (1940) has demonstrated that the irrelevant absorption is considerable in low potency oils, which has been found to hold good in shark-liver oils also (Table IV).

The values obtained on the Hilger vitamer A were in close agreement with those on the Hilger quartz spectrophotometer. A satisfactory figure can be arrived at on the former instrument from the mean of three determinations at different concentrations, at least five readings being taken at each concentration. Ewing *et al.* (1940) have reported favourably on the vitamer A. Irish (1936) and Wilkie (1937) concluded, after a collaborative study of a number of laboratories, that provided the manipulations adopted are the same, fairly concordant results can be obtained. Holmes *et al.* (1937) recommended that, in order to increase the accuracy, the vitamer A should be standardized against a spectrophotometer. It has been observed that unless care is taken to adjust the arc in line with the centre of the filter when the fluorescent lines will be of equal length, large errors will be introduced.

SUMMARY.

1. The vitamin A in commercial samples of shark-liver oil is gradually destroyed on keeping. A systematic inquiry into the causes of the loss and the methods of preservation is necessary.

2. The Pulfrich photometer has been standardized for the estimation of vitamin A in shark-liver oil by the Carr-Price reaction. The factor for converting $B_{1\text{ cm.}}^1\%$ into international units per gramme is 910 (average).

3. Provided the mean of a number of readings is taken, the Hilger vitamer A gives results as reliable as the spectrophotometer. In low potency oils there is considerable irrelevant absorption in the ultra-violet region.

ACKNOWLEDGMENTS.

The author wishes to express his grateful thanks to Mr. B. N. Banerjee and Professor V. Subrahmanyam for their kind encouragement and keen interest in the work. Thanks are also due to the Department of Industries and Commerce, Madras, for meeting half the expenses of the inquiry.

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STUDIES ON THE DESTRUCTION OF VITAMIN A IN SHARK-LIVER OIL.

Part II.

DEVELOPMENT OF ACIDITY DURING STORAGE OF LIVERS.

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The quality of shark-liver oil will depend on a number of factors, such as the condition of the liver prior to the extraction, method of extraction, extent of exposure to light and air, presence of pro-oxidants particularly metals and metallic compounds, and presence of anti-oxidants. This paper deals with the first of the factors, being a comparative study of the oils extracted from livers preserved for different periods and under different conditions.

The British Pharmacopœia (1932) specifies that the acid value of cod-liver oil should be less than 1.2. Ganpule and Sarangdhar (1943) have reported values within this limit for a number of samples of shark-liver oil extracted by them. Commercial samples of pure shark-liver oil tested in this laboratory were, however, found to have an undesirably high free fatty acid value, i.e. 5.0 to 15.0, which indicated that the livers used for the preparation of the oils were not fresh and had been preserved under unsuitable conditions. Rangaswami (1942) obtained similar high values for the acidity. The presence of free fatty acids in an oil has been claimed by some workers to have an accelerating effect on oxidative rancidity (Greenbank, 1936; Greenbank and Holm, 1924); but Lea (1938) (quoted by Brocklesby, 1941) found no such effect. Further, it is well known that vitamin A is destroyed rapidly by rancid fats (Fridericia, 1924; Powick, 1925; Wokes and Willimott, 1927; Smith, 1939). Dann (1932) observed that vitamin A was oxidized more rapidly in acids than in alcohols or esters. Hence the inquiry into the cause of high acidity and its prevention in shark-liver oil attains additional importance from a practical point of view.

Drummond and Hilditch (1930) studied the relationship between the time of storage of cod livers at a temperature not exceeding 5°C. and the quality of the resulting oil. They noted a progressive increase in the free fatty acids and colour with ageing of the livers and a progressive loss in yield of oil due to the formation of emulsions. Brocklesby (*loc. cit.*) reported that halibut livers, if not processed whilst still fresh, gave an oil that was unusually high in free acids. No decrease in the rate of production of free acids was observed by these workers when mild antiseptics such as common salt were used to preserve the livers and they concluded that the natural enzymes present in the liver tissue were responsible for the deterioration of the oils. Brocklesby and Green (quoted by Brocklesby, *loc. cit.*) found that borax (2 per cent) and formalin (0.25 per cent) kept halibut livers fairly free from bacterial decomposition for a week; in the former there was little, if any, loss in vitamin A but with formalin rather serious losses were obtained. The free fatty acid content was not determined by the authors.

The liver oil industry in India is still in its infancy and has not been developed to the same high technical standard as in foreign countries. It is, essentially, a cottage industry and a by-product of the fishing industry since the sharks are caught not so much for the livers as the body which is edible and more valuable.

To economize on labour and fuel, and ensure a high recovery of the oil, in certain areas the livers are transported to a central factory, thus causing a delay of one to three days in rendering. If the catch of sharks happens to be small, the livers are stored till a sufficiently large quantity becomes available. During the interval common salt is generally used as the preservative against putrefaction, the larger livers being chopped into small pieces before salting. Experiments described below indicate that, under the tropical conditions, this method is not suitable. Ice was not used as a preservative since it is not practicable at this stage of the industry.

EXPERIMENTAL.

A number of small, fresh shark livers (2 lb. to 3 lb.) which generally consist of two lobes were cut in the middle and the corresponding halves mixed to form two batches. The oil from one batch was extracted immediately by boiling in water in an enamelled basin for half to one hour. The contact of hot oil with air was minimized by skimming off the oil as soon as it collected on the water. The other batch of livers was preserved by adding 20 per cent of its weight of common salt. It was observed that salt generally drew out part of the water from the livers which shrivelled slightly. After a definite period the salt was washed off and the oil extracted as described above. The results of analyses of the samples of oil obtained from a number of such experiments are summarized in Table I:—

TABLE I.
Storage of livers and quality of the resulting oil.

Experiment.		Period of storage, hours.	Yield of oil, per cent.	COLOUR OF OIL.		Acid value.	Vitamin A B 1 % 1 cm.
				Yellow.	Red.		
1	A	..	Fresh	0.36	4.24
	B	..	18	11.1	3.25
2	A	..	Fresh	16	14.1	0	0.48
	B	..	42	13.5	32.1	6.6	9.55
3	A	..	Fresh	17	0.20
	B	..	66	11.8	17.34
4	A	..	Fresh	22	18.0	0	0.13
	B	..	90	12.5	22.5	9.6	19.1
5	A	..	Fresh	30	3.0	0	0.42
	B	..	240	10.8	27.0	8.7	34.75

The colour of the oil is expressed in terms of yellow and red Lovibond units for 1 cm. thickness of oil. When the oil was too darkly coloured it was suitably diluted with chloroform before measuring the colour. The vitamin A was determined by the Carr-Price reaction on a Pulfrich photometer as described in Part I of this paper (*see p. 155, this issue*). The acidity was expressed as mg. of potash per gramme of oil.

The influence on the resulting oil of intimate mixing of the liver with salt, instead of preserving it whole, was next studied. A medium sized liver (weighing 10 lb.) was thoroughly minced with a meat-mincer and a portion of it mixed with 25 per cent common salt and stored. The rest was treated immediately. At intervals a fourth of the preserved liver was extracted, no attempt being made to wash off the salt. It was observed that during the preservation

some oil oozed out from the liver. The experiment was repeated (II) with another liver (weighing 11 lb.) the concentration of salt used for preservation being 20 per cent. The results are given in Table II :—

TABLE II.

Storage of livers after mincing and quality of the resulting oil.

Experiment.		Period of storage, hours.	Yield of oil, per cent.	COLOUR or OIL.		Acid value.	Vitamin A 1 % B 1 cm.	
				Yellow.	Red.			
I	A	..	Fresh	..	15.0	0.6	0.5	32.34
	B	..	46	20.0	3.02	..
	C	..	67	21.6	18.0	2.0	3.75	31.35
	D	..	115	23.3	22.0	2.6	5.1	32.0
	E	..	234	20.0	6.15	..
Mean			..	21.2				31.0
II	A	..	Fresh	..	9.9	0.3	0.75	21.34
	B	..	46	25.6	2.10	..
	C	..	67	27.8	12.9	0.9	4.05	..
	D	..	115	28.0	20.1	2.4	5.65	20.0
	E	..	234	23.0	7.36	21.1
Mean			..	26.1				21.1

The rapid increase in free fatty acids and colour observed in Table I is reduced greatly by initial mincing of the liver and the loss of oil avoided (Table II). The increase in red units which is responsible for the dark colour was out of proportion to the increase in the yellow component.

DISCUSSION.

The usual method of preserving livers did not prevent the rapid increase in free fatty acids. If the liver tissue was disrupted to a certain extent by efficient mincing before the salt was added, the rate of development of acidity was considerably reduced. Though enzyme action did not appear to be inhibited completely, the hydrolysis proceeded much more slowly. Even then the increase was greater than that observed by Drummond and Hilditch (*loc. cit.*) with cod livers preserved at 5°C. It might possibly be due to the temperature of preservation being much higher (nearly 35°C.) and nearer the optimum for enzyme action. These workers specify that only livers preserved at 0°C. and not more than 18 hours old should be used in the preparation of medicinal cod-liver oil. Under the tropical conditions, even a few hours' delay may be expected to produce hydrolytic rancidity in the oil to the same extent as preservation for 18 hours at 0°C.

The oil from preserved livers had a dark colour compared to the golden yellow from fresh ones. The former invariably possessed an unpleasant sour odour, whereas the latter was

fishy but quite tolerable. The development of colour and unpleasant odour in minced livers was slower.

Another serious disadvantage in preserving livers is the loss of oil due to the formation of emulsions. Brocklesby (*loc. cit.*) explained that emulsification was promoted by the action of soaps formed by the combination of ammoniacal bodies and free fatty acids which are products of liver autolysis. The uniformly good recovery of oil from livers minced before preservation might be due to the fact that the salt which was not washed off prior to rendering dissolved in the water and prevented emulsification. Actually the separation of oil from the stick-water and foots was quicker and more satisfactory.

The results in Table II indicate that no vitamin A was destroyed during the preservation of livers, the oil from preserved livers having the same potency as that from fresh livers. This was observed by Drummond and Hilditch (*loc. cit.*) and Brocklesby (*loc. cit.*) also. It is possible that the differences in vitamin A noted in the first four experiments in Table I were due to the unequal distribution of vitamin A in the different parts of the liver. Popper and Brenner (1912) demonstrated that in the hypervitaminotic rat the excess of vitamin A was stored in the Kupfer cells which are not regularly distributed in the liver. The same condition might exist in shark livers also.

The results which are of practical importance clearly indicate that to produce an oil of low acidity and tolerable odour absolutely fresh livers have to be used. Under exceptional circumstances livers may be stored for a few hours in salt after efficient mincing.

SUMMARY.

Though the vitamin A potency remains practically unaffected during the preservation of livers with salt, the free fatty acid content, colour and odour of the resulting oil increase rapidly. Preliminary mincing of the livers appears to retard these undesirable changes considerably. To produce a high-grade oil livers should be rendered within a very short time after landing the sharks.

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VITAMIN C IN PINE NEEDLES.*

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ALTHOUGH vitamin C or ascorbic acid was synthesized in good yield by Reichstein and Gruessner as early as 1934, the search for rich natural sources of this vitamin is going on unabated even in the present day. The pure synthetic ascorbic acid is manufactured only in countries where the industry of fine chemicals has been well established. In countries like India, China, etc., where the fine chemical industry is practically non-existent, the manufacture of synthetic vitamins is not accomplished and hence such countries are entirely dependent upon foreign imports. After the advent of the present world war, the importation of these synthetic vitamins was naturally restricted, and the acute shortage of these valuable products has been felt. Even in Western countries where the synthetic production of vitamin C has been well established, the manufacture of vitamin C concentrates from natural sources is undertaken extensively. It should, therefore, be concluded that synthetic production is not enough to meet the demands even in such countries.

In an investigation for natural sources of vitamin C, it was discovered (Sabalitschka, 1940 ; Pyke and Melville, 1942) that rose-hips contain 100 mg. to 400 mg. of vitamin C per 100 g. Some species (*R. corifolia*) have been reported to be very much richer. Unripe walnuts (Pechniksva, 1940 ; Ranganathan, 1942) were also found to be very rich source containing 410 mg. to 1,800 mg. per 100 g. Scientists in the Soviet Union have reported that pine needles have been used for the manufacture of vitamin C concentrates.

In India among the various plant sources tested it has been found that *amla* (the Indian gooseberry) is a good source for ascorbic acid† (Damodaran and Sreenivasan, 1935). Tablets containing dried pulp of the berries have been manufactured. On account of the presence of tannin, the tablets do not appear to be palatable.

Pine needles do not appear to have been tested for vitamin C in India, probably due to the fact that they are not edible. Needles of *Pinus longifolia* are available in unlimited quantities on the lower Himalayas.

MATERIALS AND METHODS.

Pine needles were collected at Kasauli (Punjab) and the end-portions were cut off since the distal end was dried up and the proximal end was woody. The middle portions were collected and 5 grammes of a representative sample taken for the determination of vitamin C. Estimations of vitamin C were made by the specific and accurate chemical method introduced by Tillmans (1932). The technique followed was that described by Harris and Ray (1932) and Harris and Olliver (1942).

* An abstract of this paper was presented to the Indian Science Congress, January 1944.

† Dried *amla* powder which was kept for some time was tested by us and found to contain between 2 mg. and 3 mg. of ascorbic acid per gramme.

Four major precautions should be observed in order to obtain accurate results in the titration of vitamin C by this method: (1) representative sampling, (2) complete extraction, (3) prevention of oxidation, and (4) rapid performance of the titration itself (Harris and Olliver, *loc. cit.*). All these precautions were rigidly followed in our work. In addition, the extracts were also titrated against $\frac{N}{100}$ iodine as a further check on the possible presence of non-specific reducing agents. The results are given in Table I:—

TABLE I.
Vitamin C content of pine needles.

Sample number.	Mg. of vitamin C/100 g. by Harris and Ray's method.	Mg. of vitamin C/100 g. by iodine titration.
1	122	120
2	104	112
3	145	158
4	108	116
5	155	165
6	99	109

Pine needles cast from the trees were gathered from the ground and tested for vitamin C. They were found to contain only from 25 mg. and 40 mg. of ascorbic acid per 100 g. The needles of young pine trees under 4 feet high were found to be of paler green colour and were more fleshy than the needles of older trees. The vitamin C content of some representative samples from young pine plants was estimated (Table II).

TABLE II.
Vitamin C content of needles from young pine trees.

Sample number.	Dye method.	Iodine method.
1	150	108
2	142	102
3	158	164
4	164	179
5	140	148

In general, it was found that the values obtained by iodine titration were not very much higher than those obtained by the dye method. It is, therefore, likely that the reducing action of the extracts can be attributed chiefly, if not wholly, to vitamin C.

The direct titration with the dye fails to detect : (1) reversibly oxidized ascorbic acid and (2) combined ascorbic acid. It is now recognized that these two forms of vitamin C are biologically active and hence ascorbic acid does not represent the total vitamin C content. These pine needles were, therefore, tested for the presence of these two forms by the appropriate methods.

(1) *Reversibly oxidized ascorbic acid*.—This was tested for by passing H_2S through the original acid extract for 10 minutes and keeping the extract content overnight. The H_2S was then removed in a current of CO_2 applying suction at the opposite end also. This was then titrated against the dye. No increase in ascorbic acid was noticed and it was therefore concluded that no reversibly oxidized vitamin C is present in pine needles.

(2) *Combined ascorbic acid*.—The needles were ground with 50 c.c. of 0.2 per cent HCl and metaphosphoric acid, and transferred to a small wide-mouthed flask. H_2S was passed for 10 minutes and the flask was stoppered and kept at $40^\circ C$. for one hour. The solution was then squeezed through muslin. The residue was extracted with 12.5 c.c. of 20 per cent trichloroacetic acid and 5 c.c. of 20 per cent metaphosphoric acid. H_2S was passed and the flask stoppered and left overnight. H_2S was removed in a stream of CO_2 . The solution was filtered and the filtrate titrated against the dye. No increase over the original vitamin C value by direct titration was observed.

It was therefore concluded that vitamin C is present only in the reduced form in pine needles. In fact the existence of this form of combined ascorbic acid has been questioned by Harris and Olliver (*loc. cit.*) who did not find any increase in the ascorbic acid in various vegetables alleged to contain combined ascorbic acid, by the treatment described above which is designed to liberate vitamin C from its bound form.

It has been suggested that the results obtained by the chemical method may possibly be too high owing to the presence of substances other than ascorbic acid which may reduce the dye. Emmerie and van Eekelen (1934) proposed a preliminary treatment with mercuric acetate to remove such reducing substances. This modification has been considered inadvisable by Bessey (1938), McHenry and Graham (1935) and others. Under the conditions of the test, glutathione and iron salts do not interfere. Cysteine is not usually present in ordinary vegetable extracts in sufficient quantity to interfere with the test. By treatment with mercuric acetate the vitamin C is adsorbed, and hence values obtained by this method are lower than the real values. This was tried for pine-needle extract and a value of about 15 per cent less was obtained apparently due to the adsorption of the vitamin by the precipitate and not really due to the presence of cysteine. Having thus established that pine needles are rich in vitamin C (100×175 mg./100 g. of the needles), attention was next directed to the preparation of concentrates.

The water extract of the needles was found to be strongly acid (20 g. leaf extracted with 20 c.c. distilled water, the pH of the extract was about 3.5 at which vitamin C is fairly stable). Hence for the preparation of the extract on a large scale acetic acid was not found to be necessary. One thousand grammes of the needles were ground on a stone to a fine paste with a small quantity of water and the pulp pressed through muslin. This was done 4 times so as to ensure the maximum extraction of the vitamin. The volume of the extract which was about 3,000 c.c. was brought down to 500 c.c. by vacuum concentration. This concentrate was syrupy in consistency and had strong adhesive properties. The gum present in this extract was found to be water soluble and hence is different from the wood resin which is insoluble in water but soluble in alcohol. Two volumes of methyl alcohol were added to this concentrate and the gummy precipitate removed by filtration under suction. The filtrate was mixed with an equal quantity of acetone and the precipitate washed with a small quantity of methyl alcohol. The combined filtrates were concentrated and the syrupy residue, which was highly concentrated, contained about 20 mg. of vitamin C per c.c.

Vitamin C concentrates are extremely unstable and hence it is very necessary to ensure favourable conditions for stability. Vitamin C concentrate prepared by the process described,

as well as concentrated aqueous extract of pine needles containing 1 mg. of vitamin C per c.c. were stored in the refrigerator and in the incubator for a period of one month and the rate of destruction of the vitamin studied. 0.1 per cent NaHSO_3 was added as a preservative. Interference by sulphite was overcome by using 20 per cent acetone in the titration mixture as recommended by Mapson (1912). The results are given in Table III :—

TABLE III.

Deterioration of vitamin C in stored extracts of pine needles.

Period of storage in days.	VITAMIN C CONCENTRATE CONTAINING 20 MG./C.C.		AQUEOUS PINE-NEEDLE EXTRACT CONTAINING 1 MG./C.C.	
	Percentage loss at 4°C.	Percentage loss at 37°C.	4°C.	37°C.
5	10	40	8	45
10	18	78	17	80
20	42	95	45	96
30	66	100	70	100

The vitamin is destroyed more slowly at 4°C. than at 37°C. Sugars have been reported to have a stabilizing effect on vitamin C in rose-hip extracts (Wokes, 1942). The effect of the addition of 60 per cent cane sugar to the pine-needle extract was therefore studied and the results are given in Table IV :—

TABLE IV.

Deterioration of vitamin C in stored extracts of pine needles after addition of cane sugar.

Period of storage in days.	Pine-needle extract syrup stored in the refrigerator.	
	Percentage loss at	
	4°C.	37°C.
5	5	15
10	12	29
20	18	40
30	25	59
45	32	80
60	40	100

The deterioration is reduced by the addition of cane sugar but is not completely prevented. Wokes *et al.* (1943) have shown that the rate of destruction of vitamin C in rose-hip syrup is affected by exposure to air.

In order to reduce the exposure to air to a minimum, the pine-needle extract syrup was filled in sterilized ampoules with a minimum of air space and sealed. The rate of deterioration of the vitamin, in these ampoules when stored in the refrigerator as well as at 37°C., was studied and the results are given in Table V :—

TABLE V.
*Effect of exclusion of air on the deterioration of
vitamin C in pine-needle extracts.*

Period of storage in days.	Percentage loss at	
	4°C.	37°C.
10	8	20
20	13	32
30	19	45
50	28	70
70	38	90
90	48	100

When stored in completely filled ampoules, the deterioration is reduced. In all the published reports of the deterioration of vitamin C in various commercial extracts, it is not known whether oxidation of the vitamin is reversible or irreversible. If part of the destruction is due to reversible oxidation only, this loss is immaterial since it is now established that reversibly oxidized ascorbic acid is biologically as active as the vitamin itself. In order to determine whether the observed deterioration of vitamin C in our experiments was due to reversible or irreversible oxidation, the reversibly oxidized vitamin was converted to ascorbic acid by passing H_2S as described previously. The results are given in Table VI :—

TABLE VI.
*Deterioration of vitamin C in pine-needle extracts due to
reversible oxidation.*

Period of storage in days.	Total percentage loss at 4°C.	Percentage loss due to reversible oxidation.	Percentage effective loss.
10	8	5	3
20	13	8	5
30	19	10	9
50	28	12	16
70	38	15	23

The effective loss is thus about 23 per cent when the syrup of the pine-needle extract is stored in the refrigerator in sterilized and completely filled ampoules.

SUMMARY AND CONCLUSIONS.

1. Pine needles obtained from *Pinus longifolia* have been found to be rich in vitamin C (100 mg. to 175 mg./100 g.).
2. In aqueous extracts of these pine needles, vitamin C was found to deteriorate when stored at varying temperatures. Deterioration was less at the refrigeration temperature.
3. Addition of 60 per cent cane sugar reduced the rate of deterioration of vitamin C.
4. The rate of deterioration was further reduced by the exclusion of air.
5. Extracts of pine needles prepared in sugar and stored in sealed ampoules at refrigeration temperatures were found to be fairly stable. Such extracts were found to be non-toxic to rats and guinea-pigs when fed for a period of one month.
6. In view of the above findings, the possibility of obtaining fairly stable preparations of vitamin C might be considered

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NUTRITIVE VALUES OF SOME MARINE FISHES OF BOMBAY.

BY

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SCARCELY any attention seems to have been devoted to any systematic investigation of the nutritive value of the marine fishes of Bombay. The consequence of this becomes all the more striking when it is realized that this gap in our knowledge is a big handicap to any proposal to popularize the less known varieties of fishes. The nutritive value of fishes is a problem that has engaged the earnest attention of investigators in America, where there is hardly any fish the nutritive value of which is not known.

The works of Atwater (1888), Rozov (1891), White and Crozier (1911), Clark and Almy (1918), and Dill (1921) constitute an important contribution to the food value of the fishes of the United States. Professor Atwater analysed about 50 species, and his report on the chemical composition and nutritive values of edible fishes and aquatic vertebrates, published in the Report of the Commission of Fish and Fisheries, 1888, is accepted as an authoritative contribution to the knowledge of the chemical composition of American food fishes. Considerable use has been made of the data by eminent writers on the subject of food and nutrition. The causes and extent of variation in the composition of fishes were also discussed by Clark and Almy (*loc. cit.*) in their paper on 'A Chemical Study of Food Fishes', and their work forms a notable addition to the ichthyological literature.

The same cannot be said of India's fishes, though it must be admitted that some work has been done on salted and dried fish of Bombay's coastal waters by Niyogi *et al.* (1941). Greater attention has been devoted to the fresh-water fishes of Bengal by a number of investigators in that province, and their investigation constitutes a useful addition to the literature on the subject. Niyogi *et al.* have analysed only five varieties of fresh marine fishes of Bombay, and this number cannot in any way be said to be representative of the great number of fishes available for food and for other purposes. Hence, it was thought that investigation of the nutritive value of other marine fishes might help the consumption of our little known varieties. There is no question of the importance of information of this character. The possibility of making practical use of such data is almost unlimited. The knowledge will be useful to canners and manufacturers of fertilizers, fish meal and fish oil, for without a thorough understanding of the basic composition of the raw material the success of their enterprise is likely to be limited. Equally, traders in fresh fish and the great body of consumers will also benefit from such knowledge, inasmuch as they will be in a better position to appreciate the particular features and qualities of the product they buy for consumption.

METHOD OF WORK.

The edible portion of any fish is mainly its muscular tissue, and hence only the flesh separated from the skin and bones and finely minced was analysed for the purposes of the present investigation. The head, fins, skin, scales, skeletal structures and entrails of the various fishes examined were discarded. The size of a fish was one of the main factors taken into consideration in the selection of specimens for analytical work. If a fish was less than 6 inches long, several specimens of the same kind were taken until about a pound of their

muscular tissue was obtained, and this was examined. In regard to medium-sized fish a representative sample was obtained by mincing flesh from about half a dozen fish. For fish exceeding 2 feet in length analytical samples were obtained by taking pieces of flesh from different regions of the trunk and mincing them till a pulp was formed. This pulp was used for investigation.

Analysis.—The nitrogen content was estimated by Kjeldahl's method, the amount of nitrogen so obtained being multiplied by the factor 6.25 in order to obtain the total proteins. The moisture content was determined by drying a weighed sample of fresh fish to a constant weight in a steam oven at 100°C., while the quantity of ash was estimated by Stolte's dry ashing method, as applied by Tisdall and Kramer (1921). The ash was dissolved in hot 0.5 N hydrochloric acid and filtered free from insoluble matter into a volumetric flask. The residue and the platinum crucible were repeatedly washed with water and the washings passed through filter into a flask. The volume of solution was made to 100 c.c. and aliquots of this were used for the estimation of iron, phosphorus and calcium. Iron and phosphorus were estimated colorimetrically, the former by Wong's method (1928) and the latter by Briggs' (1922) modification of the Bell-Doisy (1920) procedure. Calcium was estimated volumetrically by McCrudden's permanganate method (1911-12).

Table I gives the vernacular, common English and scientific names of the fishes and the percentage of their edible portions, as well as percentages of protein, moisture, ash and other essential minerals of the edible portion. The protein content of beef, pork, mutton, goat meat and chicken was also determined for comparison with the protein contents of the fishes examined. The results of this analysis are indicated in Table II.

The gustatory quality of fish is one of the main factors which account for its universal popularity as an article of food. Its easy assimilativeness and absence of tough muscles and ligaments are other qualities that commend it to the palate. Not generally realized is the valuable quality of its protein and other essential minerals, which constitute, by far and large, some of the most essential constituents of food. Experiments have established the superiority of fish proteins and minerals to those obtained from many other sources of food. Clark and Almy (*loc. cit.*), and Dill (*loc. cit.*) have, however, shown that there is a definite seasonal variation in the composition of fishes of the same species and hence the results indicated in Table I, which refer to the months of April to October, may be regarded as representing only a general composition of the various fishes.

The main conclusion to be drawn from the table is that the protein contents of Bombay fishes vary from 15 to 25 per cent. while the contents of moisture and ash fluctuate between 70 and 80 per cent and one to 2 per cent respectively. The mineral contents of the fishes also show specific variations. There is wide variation in the percentage of edible material. The Bombay duck comes first with 78 per cent, there being very little waste. It is followed closely in the extent of flesh edibility by 'Saranga' (*Stromateus cinereus*), 'Halva' (*Stromateus niger*), 'Niwta' (*Boleophthalmus dussumieri*) and 'Boi' (*Mugil speigleri*). Analysis of these results shows that Bombay fishes compare very favourably with fishes elsewhere in the world, such as those examined by Atwater in America, and are in many instances even superior to them. A feature of Bombay fishes commented upon by several independent observers, is the extraordinary richness of protein content of the so-called cheap and despised varieties, such as 'Mushi' (*Carcharinus limbatus*), 'Pakat' (*Dasybatus uarnak*), 'Wam' (*Muraenesox talabonoides*), 'Karli' (*Chirocentrus dorab*), 'Wakti' (*Trichiurus savala*), 'Bangda' (*Scmber microlepidotus*) and 'Dagol' (*Chorinemus toloo*). They are richer in protein value than the usually esteemed delicacies such as pomfret, Rawas and Bombay duck, while they are in no way inferior in point of nutrition to the costlier flesh of the higher vertebrates. In addition to the high content of protein, almost all the fishes analysed seem to be rich in essential nutritive elements such as calcium, phosphorus and iron, the physiological value of which has been well established. Fishes are also known to possess a high iodine and copper content, both exceedingly useful, but we have not done any work to ascertain the copper and iodine content of of analysed.

TABLE I.

Analysis of fresh fish and crustacea.

Local (Bombay) name with common English name.	Scientific name.	Approximate weight of an average sized fish.	Edible portion, per cent.	PERCENTAGE COMPOSITION OF EDIBLE PORTION OF FISH.					Iron, mg.
				Moisture, g.	Protein, g.	Ash, g.	Calcium, mg.	Phosphorus, mg.	
1. Saranga (white pomfret)	.. <i>Stromateus cinereus</i>	1-3 lb.	74	71-00	16-69	1-48	250-00	21-24	3-76
2. Halwa (black pomfret)	.. <i>Stromateus niger</i>	2-5 lb.	74	70-60	18-40	2-19	158-60	16-03	4-48
3. Rawas (Indian salmon)	.. <i>Polynemus tetradactylus</i>	8 lb.	67	70-00	16-54	1-14	397-60	19-72	1-71
4. Dara (Indian salmon)	.. <i>Polynemus indicus</i>	20 lb.	65	77-38	15-50	1-29	125-30	14-77	4-50
5. Ghol (Jew fish)	.. <i>Sciaena sinia</i>	20 lb.	54	77-41	17-25	1-38	97-54	21-46	4-73
6. Surmai (seer fish)	.. <i>Cybbium commersonii</i>	12 lb.	63	78-90	17-86	1-26	345-00	17-26	4-02
7. Palla (Indian shad)	.. <i>Hilsa ilisha</i>	4 lb.	60	71-50	21-52	1-24	266-10	22-19	3-73
8. Bhing (Indian shad)	.. <i>Clupea toli</i>	5 lb.	59	78-33	17-98	1-80	567-50	23-01	2-84
9. Boi (mullet)	.. <i>Magil speijleri</i>	3 lb.	70	73-00	17-83	1-00	315-20	23-46	2-39
10. Bombil (Bombay duck)	.. <i>Harpodon nehereus</i>	3 oz.	78	89-30	9-05	0-71	334-20	18-60	1-29
11. Kat bangda (horse mackerel)	.. <i>Caranx crumenophthalmus</i>	4 oz.	62	76-50	20-15	1-26	385-60	6-00	6-56
12. Tel bangda (mackerel)	.. <i>Scomber microlepidotus</i>	3 oz.	61	74-70	19-53	1-65	778-20	48-14	8-49
13. Tarli (sardine)	.. <i>Clupea fimbriata</i>	0-75 oz.	65	77-36	18-57	1-60	1,136-00	52-66	6-68
14. Mandala	.. <i>Coilia dussumieri</i>	0-50 oz.	61	77-10	14-63	1-48	362-70	24-14	3-15
15. Mushi (shark or dog-fish)	.. <i>Carcharias limbatus</i>	8 lb. (edible size)	57	72-00	26-10	1-43	107-00	13-30	3-86
16. Kan mushi (hammerhead shark)	<i>Sphyrna blochii</i>	10 lb. (edible size)	53	75-14	23-90	1-10	118-00	15-11	4-48
17. Pakat (whip ray)	.. <i>Dasybatus uarnak</i>	10 lb. (edible size)	48	77-50	20-04	1-15	152-00	18-92	6-15
18. Wam (eel)	.. <i>Muraenesox talahonoides</i>	10 lb.	63	80-00	10-92	0-91	280-00	26-52	4-24
19. Shingala (cat fish)	.. <i>Arius dussumieri</i>	8 lb.	53	78-10	12-72	0-88	307-00	24-32	3-81

TABLE I—*concl'd.*

Local (Bombay) name with common English name.	Scientific name.	Approximate weight of an average sized fish.	Edible portion, per cent.	PERCENTAGE COMPOSITION OF EDIBLE PORTION OF FISH.				
				Moisture, g.	Protein, g.	Ash, g.	Calcium, mg.	Phosphorus, mg.
20. Dagol <i>Chorinamus tolao</i>	8 lb.	59	72.20	21.20	1.21	398.20	16.21
21. Kari (silver-bar fish)	.. <i>Chirocentrus doral</i>	1 lb.	67	76.11	19.78	1.50	1,150.00	22.39
22. Wakti (ribbon fish) <i>Trichiurus sacala</i>	1.20 oz.	62	74.70	22.01	1.88	112.80	16.00
23. Nivta (mud skipper)	.. <i>Boleophthalmus dussumieri</i>	0.50 oz.	72	76.40	19.36	1.71	313.60	22.49
24. Dhoma <i>Sciaenidae glauca</i>	1.5 lb.	51	78.31	19.02	0.94	444.00	17.72
<i>Crustacea.</i>								
25. Shevandi (lobster) <i>Panulirus ornatus</i> var. <i>decoratus</i>	Approx. no. per lb. 2	71	76.30	19.63	1.66	178.40	40.69
26. Khekda (crab) <i>Scylla serrata</i>	6	16	81.00	15.82	1.88	893.00	53.37
27. Kolambi (prawns) <i>Parapenopsis sculptilis</i>	25	52	75.00	16.17	1.00	159.80	20.84
28. Karandi (shrimps) <i>Leander styliferus</i>	400	50	77.59	19.31	1.86	168.90	15.60

TABLE II.

Analysis of meats.

Name of meat.	Protein, per cent.	Moisture, per cent.	Ash, per cent.
1. Mutton ..	20.34	73.00	1.08
2. Goat meat ..	22.50	73.40	1.21
3. Beef ..	18.28	75.15	0.70
4. Chicken ..	19.07	73.50	1.19
5. Pork ..	19.86	64.00	1.27

A factor which should contribute to the greater consumption of these neglected varieties is their availability practically throughout the year, whereas the more popular fish such as white pomfret (Saranga), black pomfret (Halva), Indian salmon (Rawas) and Bombay duck (Bombil), which are in great demand by the public, are only seasonal in their occurrence. It is curious that people otherwise generally fond of fish prefer, on account of sheer prejudice, to go without fish if their favourite varieties are not procurable on account of their being out of season. Clear evidence of the prejudice that operates against the consumption of certain fish is afforded by the neglect of dog-fish, skates, rays and mackerel, the counterparts of which in Europe and America are in great demand among the people there.

Dog-fish, skates and rays in Bombay are valued as good food by the poor only. There is no reason why the richer classes should not avail themselves of this fish to vary their menu. The flesh of these fishes possesses many favourable qualities. Their flesh is light in colour, flaky and consistent. Prepared with mayonnaise, the taste is not unlike English salmon. Dog-fish is excellent for frying. Its flesh can be removed in large fillets which are entirely devoid of bones, a distinctive advantage over other fish. It may be cooked in a variety of ways, and this is an outstanding property. Mackerel, too, which has in recent years been appearing in increasing quantities in the Bombay market, does not command the sale which its richness of protein and other contents should entitle it to. It may be said, however, of this fish that, unlike the other cheaper fishes, it is only seasonal, being available from November to April. Mackerel occurs in such copious shoals, that if perchance, by some strange freak of nature, it had to disappear from our coast, such an eventuality would strike the death-knell of the entire coastal fishing industry, for so largely does it form its mainstay.

The low cost of first-class protein obtained from dog-fish and other despised varieties as compared with the cost of protein obtained from the more popular fishes as well as meat is shown in the following table :—

Cost of a pound of protein.

							Rs. a. p.
Mushi (dog-fish) (<i>Car-</i> at $\frac{1}{2}$ anna per lb.	0 5 0
charinus limbatus).							
Pakat (skates and rays) at $\frac{1}{2}$ " " "	0 5 0
(<i>Dasybatus uarnak</i>).							
Wam (eel) (<i>Muraenesox</i> at 2 annas " "	1 4 0
talabonoides).							
Bangda (mackerel) (<i>Scom-</i> at 3 " " "	1 0 0
ber microlepidotus).							
Saranga (white pomfret) at 6 " " "	2 15 0
(<i>Stromateus cinereus</i>).							
Rawas (Indian salmon) at 5 " " "	2 12 0
(<i>Polynemus tetradactylus</i>).							
Bombil (Bombay duck) at 2 " " "	1 14 0
(<i>Harpodon nehereus</i>).							
Leg of mutton at 8 " " "	2 8 0
							(soft parts).
Pork at 9 " " "	2 13 0
Beef round at 3 " " "	0 15 0
Chicken at 12 " " "	3 8 0

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PHARMACOLOGICAL ACTION OF ALKALOIDS OF *R. SERPENTINA* BENTH.

Part I.

NEO-AJMALINE AND ISO-AJMALINE.

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INTRODUCTION.

SIDDIQUI and SIDDIQUI (1931), working on the chemistry of *R. serpentina* Benth., isolated five alkaloids from the roots of plants growing in Bihar. They divided these alkaloids into two groups :—

Group A.—Ajmaline group, white weak bases :—

Name.		Chemical formula.	Melting point.	Percentage content on weight of dry root.
(i) Ajmaline	..	$C_{20}H_{26}O_2N_2$	158°C. to 160°C.	0.10
(ii) Ajmalinine	..	$C_{20}H_{23}O_4N$	180°C. to 185°C.	0.05
(iii) Ajmalicine	250°C. to 252°C. (decomp.)	0.02

Group B.—Serpentine group, yellow strong crystalline bases :—

(i) Serpentine	..	$C_{21}H_{23}O_4N$	153°C. to 154°C.	0.08
(ii) Serpentinine	263°C. to 265°C. (decomp.)	0.08

Later, Siddiqui (1939) reported that the alkaloidal contents of the roots of plants obtained from Dehra Dun valley were different from those of Bihar inasmuch as the former contained very little of the yellow bases while the white bases differed from the ajmaline group in their chemical constitution, melting point and yield. Two alkaloids from the Dun variety were isolated by Siddiqui (*loc. cit.*) and provisionally named as :—

Name.		Melting point.	Percentage yield in whole root.	Percentage yield in root bark.
(i) Iso-ajmaline	..	264°C. to 266°C.	0.01	0.1
(ii) Neo-ajmaline	..	205°C. to 207°C.	0.1	1.0

These two alkaloids were supplied to us through the courtesy of Dr. S. Siddiqui, Ph.D., Assistant Director, Board of Scientific and Industrial Research, New Delhi, and form the subject of the pharmacological investigations described in this paper.

EXPERIMENTAL.

In all experiments, hydrochloride salts of the alkaloids, which are easily soluble in water, were used.

I. Local effects.—

On the eye.—One per cent solution of the alkaloids instilled into the eyes of rabbits, guinea-pigs and frogs, produced no signs of irritation or local anaesthesia nor was any action produced on the pupil.

On the skin.—One per cent solution of the alkaloids did not produce any irritant action on the intact skin after local application or after subcutaneous injection.

II. Action on P. caudatum.—The paramoecia were grown by soaking hay in pond-water for a week. The action produced by the alkaloids is noted in Table I:—

TABLE I.
Action on P. caudatum.

Dilution of alkaloid.	ACTION PRODUCED ON PARAMOECIA BY	
	Neo-ajmaline.	Iso-ajmaline.
1/1,000,000	Many sluggish in 42 mins.; 50 per cent die in 1 hr. ..	No change in motility in 1 hr.; only a few die during this time.
1/40,000	Most become sluggish in 11 mins.; all die in 18 mins. ..	40 per cent sluggish in 35 mins.; 20 per cent die in 50 mins.; rest alive till 1 hr.
1/20,000	All sluggish in 4½ mins.; all die in 10½ mins. About 20 per cent sluggish in 20 mins.; 50 per cent die in 31 mins.; and 80 per cent in 1 hr.
1/10,000	All sluggish in 2½ mins.; all die in 5 mins. Nearly all die in 24 mins.
1/5,000	All sluggish in 1 min.; all die in 1½ mins. All die in 8½ mins.

It is, therefore, concluded that neo-ajmaline is more lethal to paramoecia than iso-salt, the former killing 50 per cent in 18 mins. in 1/40,000 concentration while the latter is toxic in 31 mins. in 1/20,000 concentration.

III. Toxicity.—The toxicity of the alkaloids was studied in frogs and guinea-pigs and the average lethal dose was found to be:—

	FROGS.	GUINEA-PIGS.
	(Injection into lymph-sac.)	(Injection subcutaneously.)
Neo-ajmaline 0.1 mg./g. body-weight	0.065 mg./g. body-weight.
Iso-ajmaline 0.07 mg./g. " "	0.06 mg./g. " "

IV. Action on central nervous system.—

(i) *In frogs*.—The alkaloids were injected into the ventral lymph-sac and the frogs were carefully observed. Both the alkaloids produced nearly identical actions, neo-salt producing its action in a lesser dose than the iso-salt. Neo-ajmaline, in 0.01 mg./g. dose, produced mild depression of the central nervous system—reflex time was increased and motor activity diminished. In a little higher dose (0.02 mg./g. to 0.03 mg./g.) there was an initial stimulation accompanied by restlessness and tremors. This was soon followed by severe depression and paralysis of the limbs. The respiration became progressively slow and shallow. In about $\frac{1}{2}$ to 1 hour the animal began to show signs of recovery which was complete in about 2 to 3 hours. In still larger doses the stage of depression was followed by complete paralysis and death, which usually occurred in an hour's time and was due to cessation of respiration.

In decerebrated frogs the same type of action was produced except that there was no initial stimulation. In 'spinal' animals, increase in reflex time and diminution in motor activity was seen. The alkaloids have a general depressant action on the nervous system.

(ii) *In guinea-pigs*.—The action of the two alkaloids was identical, and was very similar to that produced in frogs except that there was no initial stimulation.

(iii) *In cats*.—Only neo-ajmaline was studied in cats. The action observed was nearly the same as seen in frogs and guinea-pigs. There was slight excitement in the beginning followed by marked depression.

V. Action on cardiovascular system.—

(i) *On frog's heart*.—In frogs, the heart was perfused *in situ* through a cannula introduced into the inferior vena cava. Both the alkaloids produced nearly identical actions as described below :—

In 1/100,000 dilution the alkaloids produce a decrease in amplitude, systole being less powerful; this was followed by some slowing in rate; the rhythm later became irregular due to the occurrence of auriculo-ventricular block. If at this stage perfusion was changed over to Ringer, gradual recovery to normal rate and rhythm took place (Fig. 1). In higher concentration the same type of action was produced but occurred more quickly and was more pronounced; ultimately the heart stopped in diastole. From this stage also recovery took place when perfusion of the drug was replaced by Ringer but the contractions remained feeble and the rate slow. The slowing and irregularity could not be abolished by an injection of atropine. The cardiac output too was decreased as seen in Fig. 1.

(ii) *On isolated mammalian heart*.—The isolated hearts of rabbit and guinea-pig were depressed by both the alkaloids. There was decrease in the amplitude of contractions (Fig. 2) followed by slowing and irregularity.

(iii) *Myocardiography*.—Myocardiographic records obtained in dogs and cats showed depression in auricular and ventricular contractions, the action being more pronounced on the ventricles. Later, there was slowing and partial auriculo-ventricular block.

(iv) *Action on blood vessels*.—The blood vessels of frogs and cats were perfused by a modification of Loewen-Trendelenburgh's method. The alkaloids were perfused in two different concentrations, both of them increased the output volume of the perfusate, thus showing that peripheral vasodilatation was produced.

(v) *On organ volume*.—Spleen and intestinal volume records showed diminution with fall in blood pressure (Fig. 3).

(vi) *On normal carotid pressure*.—Both the alkaloids produced fall of carotid pressure in 1 mg./kg. to 4 mg./kg. dose. The action produced by neo-ajmaline was somewhat

more lasting. Similar action was also observed in decerebrate and spinal animals. The extent of fall produced by different doses is shown in Table II :—

TABLE II.
Showing the action of alkaloids on blood pressure.

Dose, mg./kg.	Animal preparation.	FALL WITH NEO- AJMALINE ON		FALL WITH ISO- AJMALINE ON	
		Normal blood pressure, mm. Hg	Initially raised blood pressure, mm. Hg	Normal blood pressure, mm. Hg	Initially raised blood pressure, mm. Hg
1.5	Dog	17	19	22	11
2.0	"	22	20	24	25
1.5	Cat	12	5	6	..
3.0	"	21	5	5	5
2.0	Cat spinal	6	..	No action	..
4.0	" "	11	..	8	..
1.5	" decerbr.	2	..	2	..
3.5	" "	2	..	6	..

(vii) *On experimental hypertension.*—Both the alkaloids produced fall in experimentally elevated pressure (by continuous adrenaline perfusion or ephedrine injection) of dogs and cats. The fall was proportional to the initial rise (Fig. 4).

VI. *Action on respiration.*—Respiratory movements, as recorded by a tracheal cannula, showed an increase in amplitude and rate with 2 mg./kg. dose (Fig. 3). With larger doses the stimulation was soon followed by depression.

VII. *Action on uterus.*—The action of the two alkaloids was different on strips of uteri of rabbits and guinea-pigs.

Neo-ajmaline.—In concentrations of 1/100,000 to 1/20,000, it produced a marked increase in tone and amplitude of uterine contractions. Addition of atropine did not bring about relaxation. Previous paralysation of the sympathetic endings by ergotoxine did not affect the stimulant action of the alkaloids (Figs. 5 and 6).

Iso-ajmaline.—This did not produce any action in concentrations up to 1/10,000. In higher concentration (up to 1/2,500) there was mild depression (Fig. 7).

VIII. *Action on intestines.*—The two alkaloids produced depression in the rhythmic contractions of strips of intestines of rabbits and guinea-pigs (Fig. 8). The depressant effect was antagonized by pilocarpine but not by calcium.

DISCUSSION.

The pharmacological action of two new alkaloids of *R. serpentina* 'neo-ajmaline and iso-ajmaline', from Dun valley, has been investigated. From an analysis of the effects produced, it is obvious that these alkaloids have two important actions—firstly on the nervous system and secondly on plain muscles.

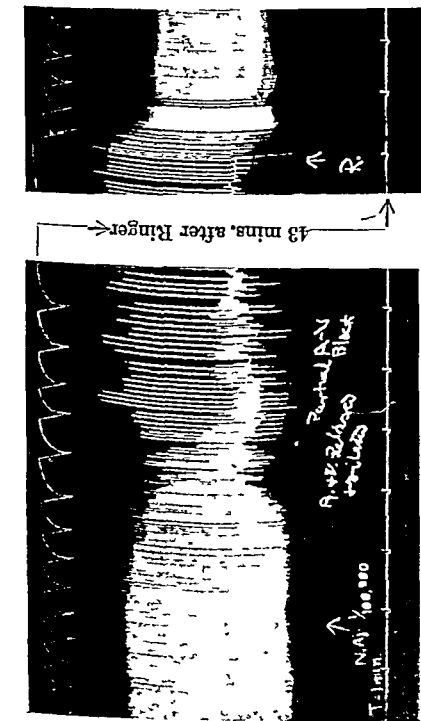
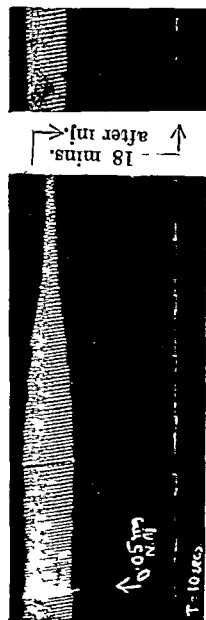
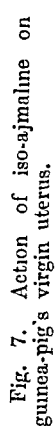


Fig. 3. Action of neo-ajmaline (2 mg./kg. dose) on normal carotid pressure, tracheal respiration and intestinal volume of a dog.



100 A3-
 7/10/98
 100 A3-
 7/10/98

Fig. 5. Action of 1/100,000 solution of neo-ajmaline on rabbit's isolated parous non-pregnant uterus.



The action of both the alkaloids on the nervous system is one of stimulation initially manifested by restlessness and tremors—followed by well-marked depression and paralysis of the various centres. The depression is particularly marked on the motor part of the cortex and medullary centres. Spinal cord is depressed but to a lesser extent. The alkaloids produced drowsiness but no actual sleep or loss of consciousness was noticed. With fatal doses the cause of death was respiratory failure, the heart continuing to beat for some time after cessation of respiration.

The action on plain muscles was depressant on the heart, blood vessels, and intestines with both the alkaloids, but they differed in their action on the uterus; one of them (neo-ajmaline) has a powerful stimulant effect, while the other (iso-ajmaline) produces mild depression of its movements.

Both the alkaloids produced fall in blood pressure in intact, spinal and decerebrate animals. The fall was particularly great in experimentally raised blood pressure. In each case with the fall in blood pressure, there was a decrease in the splenic and intestinal volume, from which it is concluded that the fall in pressure was partly due to depression of the heart. The fall in blood pressure being considerably less in the spinal animals, suggests that the fall in intact animals is partly due to the depression of the vasomotor centre.

The action on plain muscles was obviously a direct one, and was not transmitted through the adrenergic or cholinergic hormones. This is shown by the nature of the action produced which does not correspond to the autonomic supply of the viscera, and also by the fact that the effects produced were unaffected by atropine or ergotoxine. Chopra *et al.* (1942) were also unable to identify adrenergic or cholinergic effects in the alkaloids investigated by them.

We were unable to obtain the alkaloids of *R. serpentina* grown in Bihar otherwise it would have been interesting to compare their action with those described above. Judging from the published work of others (Chopra, Gupta and Mukerjee, 1933; Chopra and Chakravarty, 1941; Chopra *et al.*, 1942; Raymond-Hamet, 1940), it appears that the alkaloids under discussion differ from the corresponding alkaloids (ajmaline) of the Bihar variety in the following respects :—

(i) In being depressant to the intestines while the Bihar alkaloid (ajmaline) is stimulant.

(ii) These alkaloids, in all doses, in intact, spinal and decerebrate animals, produce fall in blood pressure, whereas the alkaloids of the Bihar variety produce rise in the intact and decerebrate animals in moderate doses, and fall in large doses in the intact and spinal animals.

SUMMARY.

The pharmacological action of two new alkaloids of *R. serpentina* (isolated from plants grown in the Dun valley, U. P.) provisionally named as 'neo-ajmaline and iso-ajmaline' has been reported in this paper.

1. Both the alkaloids are found to have a slight stimulant action on the nervous system, followed by well-marked depression.

2. Both the alkaloids are found to have a depressant action on plain muscles of the heart, blood vessels and intestines.

3. Both of them lower the blood pressure in the intact, spinal and decerebrate animals in normal condition and also after experimental hypertension.

4. Neo-ajmaline has a powerful stimulant action and iso-ajmaline has a mild depressant effect on the uterus of rabbit and guinea-pig.

5. The average toxic dose of neo-ajmaline in frogs is 0.1 mg./g. body-weight, and for guinea-pigs is 0.065 mg./g. body-weight. Iso-ajmaline is slightly more toxic. In every case death was due to respiratory failure.

ACKNOWLEDGMENTS.

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THE HYPNOTIC EFFECT OF A RESIN FRACTION ISOLATED
FROM ROOT OF *RAUWOLFIA SERPENTINA* BENTH.
OBTAINED FROM DEHRA DUN.

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INTRODUCTION.

THE root powder of *Rauwolfia serpentina* Benth. has long been used as a popular 'cure' for certain types of insanity since it produces a state of continued hypnosis. After the pharmacological investigations of Sen Gupta and Bose (1931) and Chopra *et al.* (1933), which for the first time revealed the hypotensive effect of this drug, an extract made from the root has been widely used in hyperpiesis with great benefit. Gupta, Deb and Kahali (1943) have recently reported success with the standardized extract in the treatment of different types of mental disorder. The symptoms produced after a therapeutic dose of the extract resemble those produced by the root powder, but the extract is safer as it does not depress the respiratory centre so much. Three to four hours after a therapeutic dose, preferably given in two divided doses at midday and in the evening, the patient goes into normal sleep for about 5 to 6 hours. By repeating the drug on subsequent days in adequately controlled doses a continued state of diurnal twilight sleep (semi-hypnosis) and of nocturnal sound hypnosis can be maintained. The alimentary functions of the patient improve; the bowels act regularly, appetite improves and the patient can be easily roused and coaxed to take adequate nourishment. The only drawback seems to be a profound fall of blood pressure with consequent asthenia in some ill-nourished patients.

Of the three alkaloids so far isolated by Siddiqui and Siddiqui (1931) in pure form, Chopra and Chakravarty (1941) showed that ajmaline and serpentine were definitely stimulant rather than sedative. They, however, thought it likely that serpentinine was sedative, but the later work of Chopra *et al.* (1943) did not confirm the sedative action of serpentinine, which, in their opinion, was a medullary stimulant like the other two known alkaloids of *Rauwolfia serpentina*. They found the total alkaloids fraction, as well as a new fraction of total alkaloids from which the convulsant ajmaline, serpentine and serpentinine were separated, to be hypnotic. The hypnotic dose of both the fractions was 0.05 mg. to 0.08 mg. per gramme body-weight equivalent to 50 mg. to 80 mg. per kilogram. This dose is equivalent to 10 c.c. to 16 c.c. of the standardized extract with 0.5 per cent alkaloidal content, the usual therapeutic dose of which is only 1 c.c. in man. Chopra *et al.* (1943) also found that the extract made from the Bihar plant was more sedative than the extract made from the Dehra Dun plant. Gupta and Kahali (1943) found that the hydrochloride of the purified total alkaloid of the Dehra Dun plant had no sedative action; the total alkaloidal hydrochlorides of the Bihar and the Bengal plants were sedative, and it was these two alkaloidal hydrochlorides which also gave an opaque solution in water. This naturally raised a suspicion regarding the absolute purity of the isolated samples of the total alkaloids from the Bihar and Bengal varieties. The sediment, which always deposited when a solution of the Bihar and Bengal alkaloidal hydrochlorides were allowed to stand, had a strong resemblance to the 'resin' which is present in the root

of the plant. It was, therefore, thought worth while to investigate the pharmacology of this 'resin'. Previously Sen Gupta and Bose (*loc. cit.*) had reported that, except for a slight action on the uterus, the resin of *Rauwolfia serpentina* was inactive. The resins, moreover, are generally believed to be inactive, but the sedative action of Cannabinol, the active principle of *Cannabis indica* which is a resin, encouraged us to investigate the pharmacological action of the *Rauwolfia* resin.

CHEMISTRY.

The 'resins' were separated from the alcoholic extract of *Rauwolfia serpentina* with dilute hydrochloric acid and washed free from all traces of alkaloid. After a few preliminary biological experiments, it was found desirable to separate the 'oil' from the crude 'resin' and to investigate their actions separately.

Preliminary experiments on animals proved that the hypnotic principle was associated with the oil-free petroleum-ether-insoluble fraction of the crude 'resin'. This petroleum-ether-insoluble fraction could be further separated into alcohol-soluble and alcohol-insoluble fractions. Animal experiments proved that the marked hypnotic effect was present in the alcohol-soluble fraction. The 'oil' has a bitter taste. The active 'resin' fraction is amorphous and tasteless. The relative proportion of the various fractions are:—

Crude 'resin' (petroleum-ether-soluble 'oil', 54 per cent, petroleum-ether-insoluble 'resin', 46 per cent) forms about 0.9 per cent of the standard extract. But of the petroleum-ether-insoluble resin fraction about half is alcohol-soluble and pharmacologically active.

EXPERIMENTAL.

Various laboratory animals, frogs, albino rats, guinea-pigs, rabbits and cats, were used. Rats and cats were found to be most suitable for experiments on hypnosis with the *Rauwolfia* resin fraction. The behaviour of these animals, their activity, rest, sleep, and state of digestion and nutrition, were observed for at least a week, before they were given the test fractions. The drug was given by stomach tube or was injected intraperitoneally. Given by the stomach the drug was suspended in water; for intraperitoneal injection it was first dissolved in alcohol or suspended in physiological saline made alkaline by the addition of sodium bicarbonate. In a few experiments the test fraction was given intramuscularly suspended in olive oil.

The experiments can conveniently be classified into 7 series:—

First series.—In the first series of experiments, the following three fractions separated from the root of *Rauwolfia serpentina* from Dehra Dun, and the standard alcoholic extract were given by stomach tube to a group of rabbits and cats. The fractions are:—

Fraction I.—Water soluble, free from alkaloid and 'resin'.

Fraction II.—'Resin', free from water-soluble fraction I and alkaloid, in alcoholic solution in concentration (0.9 per cent) equivalent to the 'resin' strength of the standard extract.

Fraction III.—Purified alkaloidal hydrochloride free from fractions I and II.

The doses used varied from 4 to 10 times the human therapeutic dose, i.e. 1 c.c. per 60 kg. or 1/60 c.c. per kg. body-weight calculated on the basis of the standard alcoholic extract. As all the three fractions in solution were made so as to conform to the respective strengths of the fractions present in the standard extract, the doses for all the three fractions were the same and equal to the standard extract. The alcoholic preparations were evaporated to dryness and the dry residues were suspended with mucilage before feeding the animals by the stomach tube.

Results: (a) The sedative and hypnotic effect was obtained with the standard extract and the 'resin' preparations only. In smaller doses the effect of the 'resin' was more potent than that caused by a similar dose of the extract, while in higher doses the effect caused by

the extract was more potent than that caused by the 'resin'. (b) The water-soluble fraction had no effect. (c) The alkaloid made the animals more restless. (d) A mixture of alkaloids and resin in the biggest dose was more effective than a similar dose of resin only.

Second series.—Albino rats (150 g. to 200 g.) were used. The 'resin' (dissolved in alcohol) and the alkaloidal hydrochloride (in watery solution) were given intraperitoneally. Control experiments were done to observe the effect of alcohol alone. The dose used was 1 mg. to 10 mg. per kg. body-weight.

Results: (a) The sedative and hypnotic effect was obtained with the 'resin' preparation only, the injection of which apparently caused pain. (b) Alkaloidal hydrochloride made the animals rather restless. (c) Alcohol in the quantity used had no effect.

Third series.—The crude 'resin' fraction was obviously a mixture and contained an oily substance which was extracted with petroleum ether. The 'resin' fraction thus separated from the 'oil' could be further separated into alcohol-soluble and alcohol-insoluble fractions. The effects of these three fractions, e.g. 'oil', alcohol-soluble resin, and alcohol-insoluble resin, were investigated on albino rats (150 g. to 200 g.) and cats (2 kg. to 2.5 kg.). The drugs were given intraperitoneally or intramuscularly. For the intraperitoneal injection the fractions were either dissolved in alcohol or suspended in normal saline made alkaline with sodium bicarbonate. For the intramuscular injection the fractions were mixed with (or suspended in) olive oil. The volume of the injection in all cases did not exceed 0.5 c.c. The dose of the drug was 1 mg. per kg. body-weight.

Results: (a) The sedative and hypnotic effect was produced by the alcohol-soluble resin fraction only. (b) The oil was an irritant and caused pain but partly quietened the animal too. (c) The alcohol-insoluble fraction had no effect. (d) The effect with oily suspension given intramuscularly was much delayed. Absorption into the systemic circulation seems to be delayed from oily suspension. Absorption from the peritoneum cavity was quicker than from the muscles.

Fourth series.—Cats between 2 kg. and 3 kg. body-weight were used for these experiments. The purified alcohol-soluble resin was injected intraperitoneally either in alcoholic solution or in suspension of alkaline physiological saline. The dose used was 1 mg./kg. The protocol of only one experiment is given below, the results in other experiments being similar.

6th July, 1943.—Cat no. 1 weighing 2.21 kg. (under observation for a fortnight) was given 3 mg. of purified resin suspended in alkaline physiological saline (1 c.c.) at 12 noon. At 1-30 p.m. animal quite awake; 2 p.m. food was given and eaten by the animal as usual; 3-45 p.m. animal dozing; 4-30 p.m. the animal sleeping.

7th July, 1943.—The animal sleeps off and on.

8th July, 1943.—The animal sleeps off and on.

9th July, 1943.—The animal sleeps off and on; when awakened it stretches itself and tries to stand up and walk; it can walk but has a tendency to fall on one side and looks for support against which it stands and gradually lies down to sleep. Appetite is very good. Stools soft.

10th July, 1943.—More or less in the same state as on 9th July, 1943, but is recovering.

11th July, 1943.—Animal quite recovered.

On the same day another cat (no. 2), 1.95 kg., was given 3 mg. of alcohol-insoluble resin suspended in alkaline physiological saline intraperitoneally at 12 noon. At 3-45 p.m. it was dozing; 4-30 p.m. dozing. On 7th July, 1943, and subsequent days—normal.

Results: The purified alcohol-soluble resin is hypnotic, while the alcohol-insoluble fraction has no sedative action. Similar hypnotic and sedative effects were obtained in cats by feeding the 'active' resin by stomach tube. Repeated doses for 2 days produced a continued sleepy state for 3 to 4 days.

The resin when injected into the ventral lymph sac diminished the usual spontaneous activity of frogs (*rana*).

Fifth series.—In this series the resin in alcoholic solution was given intraperitoneally to cats in 1 mg./kg. doses in the morning. Three hours after, the animal was anaesthetized with ether. It went under anaesthesia without any struggle in a very short time, and the quantity of ether required to anaesthetize the animal and to keep it under anaesthesia was only about half that usually required.

Similar synergism was shown with chloralose. After resin given intraperitoneally, the usual anaesthetic dose (0.1 mg./kg.) of chloralose intravenously could be halved (0.05 mg./kg.). The anaesthetic oral dose of chloralose could also be similarly reduced. It should be remembered, however, that the effect of the purified resin becomes obvious only after 3 hours or more.

Sixth series.—Guinea-pigs, rats and cats were used to find if the purified resin would antagonize picrotoxin. The resin antagonized the effect of picrotoxin in all these laboratory animals, the antagonism being most obvious with rats.

The protocol of one series of experiments is given below: Two series of 3 albino rats each were used. Series A received picrotoxin (1 mg./kg.) subcutaneously. Series B received purified resin (1 mg./kg.) intraperitoneally; and 3 hours after picrotoxin was given.

Series A.—		Rat 1 (85 g.)		Rat 2 (60 g.)		Rat 3 (53 g.)
Time.			Time.		Time.	
12-55 p.m.	Picrotoxin 0.08 mg. subcut.		1-4 p.m.	Picrotoxin 0.06 mg. subcut.	1-9 p.m.	Picrotoxin 0.05 mg. subcut.
1-19 p.m.	Definite convulsion.		1-39 p.m.	Occasional head convulsion.	1-30 p.m.	Definite convulsion.
1-40 p.m.	Second fit lasting 1 min.		1-50 p.m.	Occasional head spasm.	
Series B.—		Rat 1 (83 g.)		Rat 2 (63 g.)		Rat 3 (50 g.)
Time.						
12-10 p.m.	Resin 0.08 mg. i.p.			Resin 0.06 mg. i.p.		Resin 0.05 mg. i.p.
3-28 p.m.	Quiet, sleeping.			Quiet, drowsy.		Quiet, sleeping.
	Picrotoxin 0.08 mg. subcut.			Picrotoxin 0.06 mg. subcut.		Picrotoxin 0.05 mg. subcut.
4-30 p.m.	No convulsion; sleeping.			No convulsion.		No convulsion; sleeping.

Seventh series.—In this series of experiments the general pharmacological action of the resin fraction was investigated.

Local effect.—Resin 'oil' dropped into the eyes of rabbits caused congestion of the conjunctiva lasting for 2 to 3 hours. The purified oil-free resin suspended in olive oil dropped into a rabbit's eye caused only slight congestion.

Cardiovascular effect.—The effect of the purified 'resin' was studied in cats under anaesthesia with chloralose or ether with or without previous sedation with resin. The resin in 1 mg./kg. dose caused no fall of blood pressure; on the contrary it caused a slight persistent rise of blood pressure.

Intestines.—The purified resin increased the tone and rhythm of the intestine in cats under anaesthesia with ether preceded by resin intraperitoneally as also in intestinal strip of guinea-pigs suspended in Fleisch's solution.

Uterus.—The resin increases the tone of the uterine muscle in cats under anaesthesia and also in isolated uterine strips of virgin guinea-pigs suspended in Fleisch's solution. The effective dose of resin for the intact animals in these experiments was 1 mg./kg. and the minimum effective concentration of resin in the bath was 1 in 80,000.

DISCUSSION.

In all the animals used in these experiments—frogs, guinea-pigs, rabbits, albino rats and cats—the petroleum-ether-insoluble but alcohol-soluble fraction of the crude 'resin' of *Rauwolfia serpentina* Benth., Dehra Dun variety, caused sedation and hypnosis, the dose varying with animals. The effective dose for cats and rats is 1 mg. per kg. body-weight. The sedative and hypnotic effect is delayed and begins to be evident only 3 to 4 hours after the test dose has been given by mouth or intraperitoneally; even after intravenous injection the action is delayed. The sedative effect prevents picrotoxin convulsions. The resin also acts synergistically with chloralose and ether.

The 'resin' content of an effective dose of the standard extract was equal to the effective dose of the 'resin'. In smaller doses the 'resin' was found to be more effective than an equivalent dose of the standard extract. This may be due to the stimulating effect of ajmaline, serpentine and serpentinine (Chopra *et al.*, 1943) antagonizing the sedative action of the 'resin'. The more hypnotic effect of the larger doses of the extract may be due to the fact that the fall of blood pressure induced by these bigger doses enhances the sedative effect of the resin. The standard extract, both in experimental animals and in clinical practice (Gupta, Deb and Kahali, *loc. cit.*), produces a hypnotic effect after a delay of 3 to 4 hours. The purified resin induces a sedative effect also after a delay of 3 to 4 hours. The reason of the delayed action is not yet clear. It may be that a secondary product formed in the body is responsible for the sedative effect while the 'resin' itself may produce immediate pharmacological effects such as slight rise of blood pressure and stimulation of simple muscle fibres, as, for example, in the intestine and uterus.

The fact that the effective dose of the standard extract, and of the active 'resin' fraction used in these experiments are equal, that the nature of the hypnosis produced by both is similar, and the induction of sedation in both is delayed for 3 or 4 hours, suggests that the hypnotic effect of *Rauwolfia serpentina* Benth. obtained from Dehra Dun, is due mainly to the oil-free alcohol-soluble 'resin' fraction of the crude 'resin' present in the plant. The 'reeling' effect induced by the resin points to the midbrain centres being affected by its action. The nature of the hypnosis and its similarity to natural sleep suggest that the site of action may be the hypothalamic centres.

The hypotensive action of *Rauwolfia serpentina* Benth. has been proved to be due to alkaloids. The results of the work reported here suggest that the hypotensive and the hypnotic actions of *Rauwolfia serpentina* Benth. obtained from Dehra Dun are due to separate groups of chemical substances—alkaloids and oil-free 'resin' respectively. These can be easily separated and made available for separate selective clinical use.

SUMMARY.

1. From the crude 'resin' of *Rauwolfia serpentina* Benth. a fraction insoluble in petroleum ether but soluble in alcohol has been isolated. This is pharmacologically active.

2. In experimental animals (cats, rabbits, rats, frogs, guinea-pigs) it produces a sedative and hypnotic effect very similar to that produced by the standard extract of *Rauwolfia serpentina*.

3. The sedative action induced by the standard extract and also by the alcohol-soluble fraction described commences about 3 to 4 hours after administration and persists for more than 24 hours.

4. The 'resin' or alcohol-soluble fraction does not cause a fall of blood pressure but stimulates the simple muscle of the intestine and uterus. This action is produced immediately.

5. The petroleum-ether-soluble 'oil' obtained from the crude 'resin' prepared from *R. serpentina* has irritant properties.

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QUANTITATIVE ESTIMATION OF COMMON TRIHALOGEN VOLATILE ANÆSTHETICS IN BLOOD AND TISSUES OF ANIMALS.

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IN 1860, Lallemand, Perrin and Duroy determined for the first time the concentration of chloroform in blood and tissues of dogs killed by the anæsthetic. They determined the concentration of chloroform from the amount of hydrochloric acid formed in the experiment. In 1883, Grahart and Quinquad carried out the estimation of chloroform in blood by removing the blood in the absence of air and subsequently distilling it under vacuum and the quantity of the anæsthetic in the distillate was estimated by the reduction of Fehling's solution. Pohl (1890) determined the chloroform content of blood by passing the vapour over heated magnesia and determined the chloride produced by titration with silver nitrate. In 1906, two French workers, Mansion and Nicloux, employed the Dumas reaction for determining the concentration of chloroform. The error of this method according to one of the authors averaged to 2 per cent, while Tinof and Mansion showed that 96.1 per cent of the total chloroform added can be recovered from blood when treated as above. In 1906, Buckmaster and Gardner described the method of estimation of chloroform in the blood by calculating the difference in the chlorine content of the blood before and after anæsthesia. They found that the concentration of chloroform in the blood at the time when conjunctival reflexes just disappear varies between 16 mg./100 g. and 31 mg./100 g. of blood. They have also determined by this method the partition coefficient of chloroform in the plasma and the r.b.c., and the lethal concentrations of chloroform for the animals. All these methods are laborious and are not delicate. Fujiwara (1914) found that chloroform in presence of an alkali gives a pink colour with pyridine and that the method is so sensitive that it can detect chloroform in a dilution of one in a million. Later, Ross (1923) reported the same test indicating 'the independent discovery that substances containing R-C (Halogen)₃ groups (chloroform, bromoform, chloretone, chloral, iodoform, etc.) give a pink or red colour to pyridine when mixture is heated in presence of NaOH. Fujiwara applied the test to extracts and distillates of animal fluids and tissues attempting to estimate the amount of chloroform present by the depth of colour produced. This work of Fujiwara gave Cole (1926) an idea to utilize the method for the determination of chloroform in the tissues of animals anæsthetized with chloroform. The method used by Cole for determining the concentration of the anæsthetic in the tissues was as follows: The tissues were minced with scissors and submerged immediately in acidified distilled water as suggested by Fujiwara. It is kept for one hour and then taken for test. If an appreciable quantity of blood is present it interferes with the method of estimation. However, the results obtained by Cole in these experiments were of qualitative nature and need improvement for reliable quantitative estimations.

Daroga *et al.* (1941) found that if a small quantity of acetone is used in the Fujiwara reaction the colour obtained is stable for a period varying from 17 to 24 hours. They introduced this modification to estimate the percentage of carbon tetrachloride in air and in soil. They also found that if the concentration be plotted against the depth of the colour the graph is a straight line showing thereby that the colour is proportional to the amount of chloroform present. (In the case of soil they used a method of distillation.)

All the above gravimetric methods suffer from the drawbacks that the methods are laborious and not very delicate. The Fujiwara reaction applied by some workers suffers from the drawback that the colour obtained is not quite clear due to the presence of protein, etc., in tissues and blood and that the colour of the blood interferes with the method of estimation.

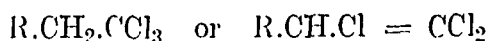
To overcome these difficulties, the present author modified the method by introducing air distillation in the Fujiwara reaction. In this method the blood is air-distilled and the distillate is absorbed in pyridine. The distillation is continued for an hour and a half and then the blood is heated so as to drive off any chloroform vapour that is remaining in the blood. The pyridine which has absorbed all the chloroform present is then diluted and the amount of chloroform or the trihalogen compound present is now estimated by the Fujiwara reaction comparing the colour obtained with a known standard. As a check on the method, a known quantity of chloroform was added to 5 c.c. of blood and the quantity was estimated by this method. It was found that the quantity added could be estimated with precision. Other trihalogen, dihalogen and monohalogen compounds were then tested by the same method. Table I gives the results obtained with different compounds :—

TABLE I.

Showing the various compounds containing chlorine tested by the modified Fujiwara reaction and the colour obtained.

Experiment number.	Name of drug.	Formula.	Concentration.	RESULTS.
1	Chlorobenzene C_6H_5Cl	10 mg./100 c.c.	No coloration.
2	Trichlorobenzene $C_6H_2Cl_3$..	"
3	Trichlorophenol $C_6H_2Cl_3$..	"
4	Ethyl chloride C_2H_5Cl	10 mg./100 c.c. approximately.	Orange colour obtained.
5 C_2H_5Cl	20 mg./100 c.c. approximately.	Orange colour deepens.
6	Parachlorometacresol	.. C_7H_7OCl	10 mg./100 c.c.	No colour.
7	Benzoyl chloride C_7H_5OCl	..	"
8	Benzyl chloride C_7H_7Cl	..	"
9	Benzyl bichloride	.. $C_7H_6Cl_2$..	"
10	Chloracetone C_3H_5OCl	..	"
11	Trichloroacetic acid	.. $C_2HO_2Cl_3$..	Pink coloration.
12	Chloral hydrate $C_2H_5O_2Cl_2$..	"
13	Iodoform CHI_3	..	"
14	Trichlorethylene $CHCl=CHCl$..	"
15	Chloreton $C_3H_5OCl_2$..	"
16	Carbon tetrachloride	.. CCl_4	..	"
17	Chloroform $CHCl_3$..	"

Table I shows that compounds of the following structure respond to the test :—



where R may be aromatic or aliphatic nucleus. It was further found that halogen attached to the nucleus in an aromatic compound does not answer the test, e.g. trichlorobenzene or trichlorophenol gave negative results. C_2H_5Cl gives an orange colour with pyridine which deepens on addition of more C_2H_5Cl . The test is also answered if instead of pyridine some of the derivatives of pyridine are used. As chemically pure pyridine was not available trials were made with an impure product and it was found that the results obtained were quite satisfactory.

METHOD.

To a test-tube $1\frac{1}{2}$ " diameter containing 40 c.c. of distilled water and 5 c.c. of 0.05 per cent saponin solution, 5 c.c. of blood from an animal anaesthetized with chloroform or trichlorethylene is added. The blood from the animal is withdrawn when the conjunctival reflexes just disappear. The test-tube is fitted with a tightly fitting rubber cork through which two

glass tubes of about 3 mm. diameter pass, one serving as an entry for air and the other as an exit for it. The exit tube is bent twice at right angles. The other end of the exit tube passes through another cork fitted to a similar test-tube containing 20 c.c. of pyridine. The exit tube dips into the pyridine in the second tube. The pyridine in the test-tube is always maintained at low temperature by immersing it in ice. The rubber cork of the second tube holds a condenser through which ice-water is circulated. The exit of the condenser is passed through rectified spirit which acts as a trap. All the connections are made air-tight except the last exit. A current of air at the rate of 15 c.c. per minute is allowed to bubble through the first test-tube containing blood. The quantity of chloroform or trichlorethylene present in 5 c.c. of blood is thus carried along with the air current and is absorbed in the pyridine which is constantly maintained at 0°C. The current is passed for 1½ hours and after this period the test-tube containing the hæmolysed blood is heated to 80°C. by gentle heat while the air current continues to pass. The flow of air is now continued for one hour more after which period the apparatus is disconnected. The rectified spirit trap is always tested for the presence of the anæsthetic and it has been found that as long as the pyridine test-tube and the condenser are properly cooled no anæsthetic could be detected in the trap. The pyridine in the test-tube which now contains chloroform or trichlorethylene is taken for qualitative and quantitative estimations.

The pyridine in the test-tube is diluted 1 to 4 with fresh pyridine. 5 c.c. of this diluted pyridine are transferred to a long, narrow test-tube containing 10 c.c. of 20 per cent NaOH and then lightly plugged with cotton-wool. The test-tube is now heated by dipping it into an actively boiling water-bath where it is kept for 1½ minutes with constant shaking. It is then cooled in the ice and the supernatant clear-coloured pyridine layer is transferred to a test-tube. The colour of this pyridine is compared in a Leitz colorimeter with a standard solution of the anæsthetic in pyridine treated in the same manner as above. The concentration of the standard is usually maintained at 1·2 mg./100 c.c. or thereabout. The colour is stable for half an hour if it is kept cold and no acid vapours are allowed to come in contact with it.

The concentration of the anæsthetic in the tissues is estimated by finely mincing the tissues and grinding them in a mortar and pestle, the whole operation being done under cold acidified distilled water. The acid water along with the suspended tissues is then transferred to a wide test-tube and the procedure described above is followed.

RESULTS.

The method was standardized by estimating a known quantity of chloroform or trichlorethylene added to the blood. Table II shows some of the results obtained and the variation between the actual quantities added and those that are estimated :—

TABLE II.

Showing the recovery of anæsthetics added to blood.

Name of anæsthetic.	Experiment number.	Quantity added.	Quantity estimated.	Variation.
Trichlorethylene	1	1·4 mg./200 c.c.	1·25 mg./200 c.c.	—0·075 mg./100 c.c.
	2	2·8 mg./100 c.c.	3·08 mg./100 c.c.	+0·28 mg./100 c.c.
	3	70 mg./100 c.c.	68 mg./100 c.c.	—2·0 mg./100 c.c.
Chloroform	4	1·125 mg./100 c.c.	1·325 mg./100 c.c.	+0·2 mg./100 c.c.
	5	15 mg./100 c.c.	15·6 mg./100 c.c.	+0·6 mg./100 c.c.
	6	75 mg./100 c.c.	75 mg./100 c.c.	Nil.

The above method has been utilized to determine the concentration of chloroform and trichlorethylene in the blood and tissues of various animals. Table III gives the results obtained as compared with those obtained by other workers on dogs anæsthetized with chloroform where the determinations were carried out by gravimetric and volumetric methods :—

TABLE III.
Showing the concentration of chloroform in blood in mg. per 100 c.c.

			Tissot.	Nieloux.	Meyer and Gottlieb.	Buckmaster and Gardner.	AUTHOR'S RESULTS. Chloroform. Trichlorethylene.	
Anæsthesia	34-40	50	35	16-31	17-32	20-37
Lethal	59-105	69-73	58	61-69	60-65	100-110

The results obtained by the author are thus in close agreement with those obtained by Buckmaster and Gardner (*loc. cit.*). The time required in all experiments to determine the blood concentrations is considerably less than that of the above authors, and no complicated arrangements are involved in the process.

A set of experiments was done with a view to find out the concentration of the anæsthetic in the blood of the animals anæsthetized with the two anæsthetics. Table IV gives the actual concentrations of the anæsthetic required to bring the various animals under anæsthesia :—

TABLE IV.
Showing the concentrations of the anæsthetics in different animals.

Name of anæsthetic.	Experiment number.	Dogs.	Rabbits.	Guinea-pigs.	Cats.
Chloroform	1	27.8 mg./100 c.c.	25 mg./100 c.c.	26.6 mg./100 c.c.	..
	2	18.5 mg./100 c.c.	34 mg./100 c.c.
	3	18.8 mg./100 c.c.	36.8 mg./100 c.c.
	4	28.6 mg./100 c.c.
Trichlorethylene	1	24 mg./100 c.c.	23 mg./100 c.c.	14 mg./100 c.c.	25.32 mg./100 c.c.
	2	28 mg./100 c.c.	23.5 mg./100 c.c.	17 mg./100 c.c.	..
	3	37 mg./100 c.c.	24 mg./100 c.c.	17.4 mg./100 c.c.	..
	4	..	28 mg./100 c.c.	17.6 mg./100 c.c.	..

Similar determinations to find out the concentration in the tissues of animals were undertaken and Table V gives the concentrations of the anæsthetic in the blood, brain and liver of guinea-pigs anæsthetized with trichlorethylene and also that of a dog anæsthetized with the same anæsthetic.

TABLE V.

Showing the concentration of trichlorethylene in blood, brain and liver of guinea-pigs and dog.

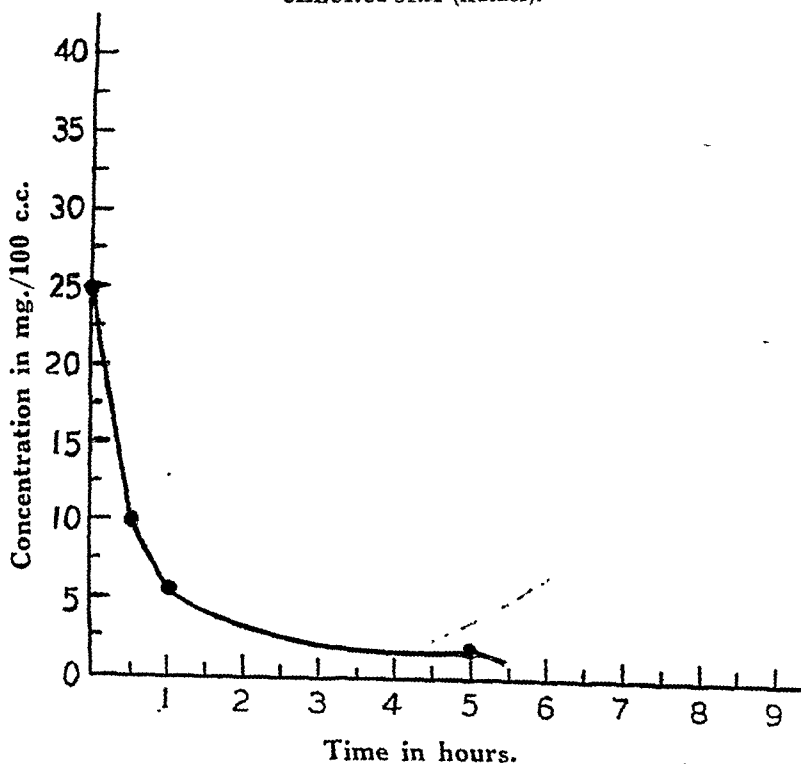
		Guinea-pigs.			Dog.
		1	2	3	
Blood	14	17.6	17	28
Liver	15.5	..
Brain	28	37.6	28.4	47

(The non-availability of large quantities of trichlorethylene prevented more extensive observation on dogs.)

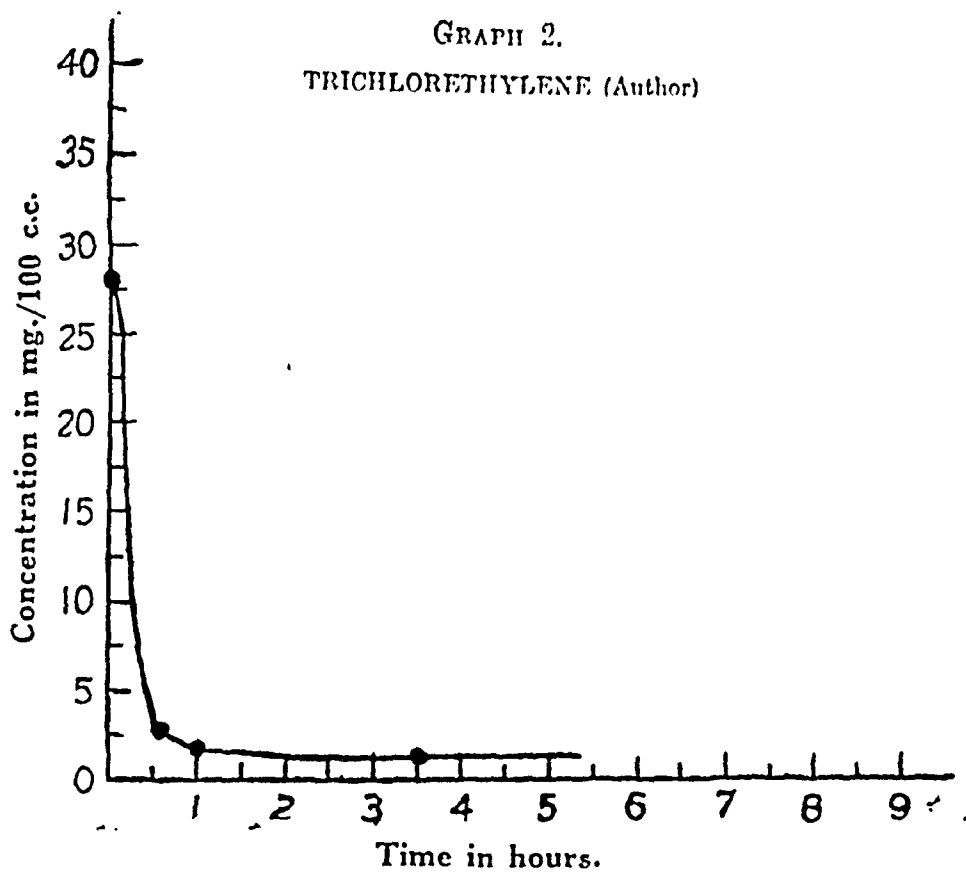
The concentration of the anæsthetic in the blood was determined at various intervals after the discontinuation of anæsthesia. Nicloux found that the concentration of the anæsthetic in the blood was reduced to half that at the time of anæsthesia after 5 minutes; 3 hours after 7 mg./100 c.c. were found in the blood and after 7 hours the blood was free from chloroform. Tissot found that immediately after cessation of anæsthesia the concentration in the blood was 53.1 mg./100 c.c. and that within 2 hours it has completely disappeared. In order to verify the results obtained by these workers the author estimated the concentration of both chloroform and trichlorethylene (a new general anæsthetic which was being investigated) in the blood at intervals after the termination of the anæsthesia. Graphs 1, 2 and 3 show the results obtained by him as compared with those obtained by Nicloux:—

GRAPH 1.

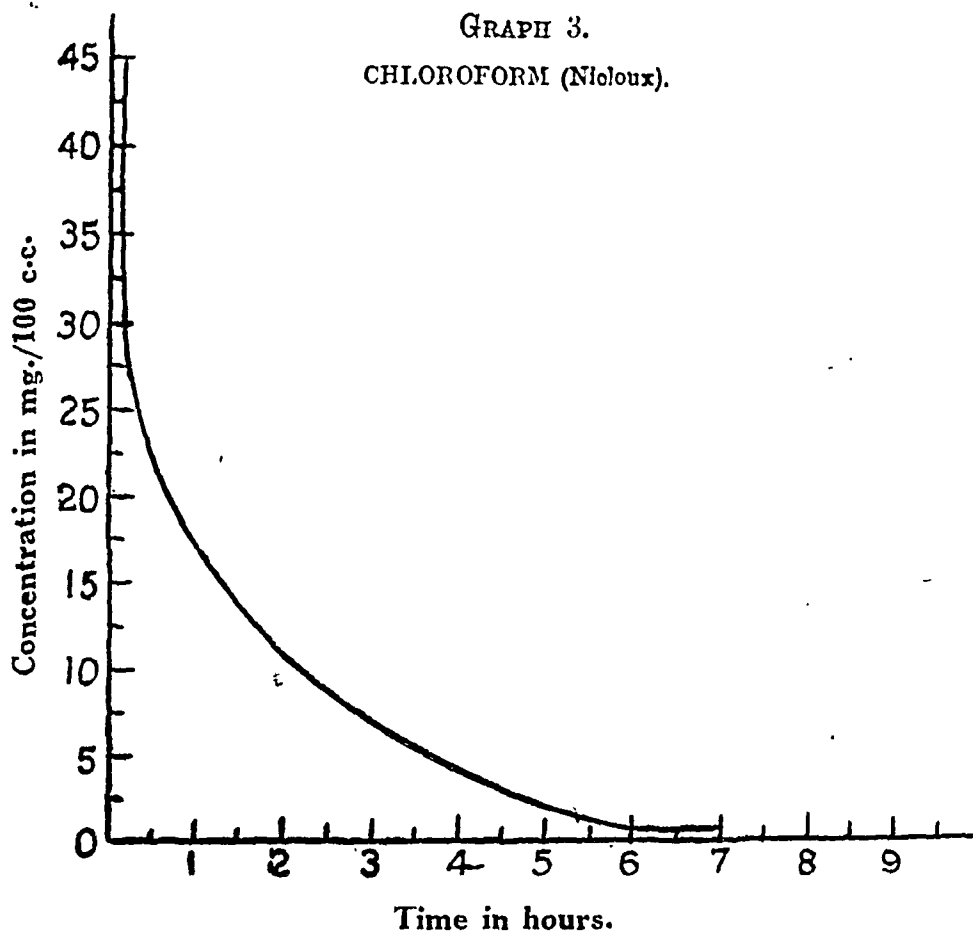
CHLOROFORM (Author).



GRAPH 2.
TRICHLORETHYLENE (Author)



GRAPH 3.
CHLOROFORM (Nicoloux).



The curves obtained for the disappearance of the anæsthetic were similar in character to Nicloux's curve but free more steeply.

SUMMARY.

A sensitive method for the determination of small quantities of trihalogen anæsthetics in the blood by air distillation has been described. The anæsthetic and the lethal concentrations of trichlorethylene and chloroform were determined for various animals. The results obtained by this method were found to agree with those obtained by Buckmaster and Gardner for chloroform. The same method was also used to determine the anæsthetic concentrations in various organs, e.g. liver and brain. The rate of disappearance of the two anæsthetics after cessation of anæsthesia was also determined.

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ATTEMPTS TO TRANSMIT HUMAN LEPROSY TO SPLENECTOMIZED MONKEYS.

BY

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INTRODUCTION.

SINCE the discovery of Hansen's bacillus in 1874 numerous attempts have been made to produce human leprosy in experimental animals; monkeys, rabbits, guinea-pigs, rats, mice, etc., have all been used for this purpose. Certain workers have claimed successful transmission to some of these experimental animals, but it is very doubtful whether human leprosy has ever been transmitted to any of the experimental animals. The latest claim of the successful transmission was that of Adler (1937) who reported that in the Syrian hamster he had after all found an animal susceptible to human leprosy. However, Dharmendra and Lowe (1940) were not able to confirm the findings of Adler. They obtained no macroscopic or microscopic evidence of a generalized infection in Syrian hamsters following an inoculation of leprous material, either by inserting a piece of leprous nodule under the skin or by intraperitoneal injection of an emulsion prepared from the nodule.

Monkeys have been used by various workers for experimental transmission of human leprosy. A few workers have reported the production of secondary lesions (lesions away from the point of inoculation), and generalized infection, but this work has never been confirmed by others. Most of the workers have obtained localized retrogressive nodules at the point of inoculation; the nodules regress after a few weeks of their appearance, and are not followed by generalized infection. Some workers believe that these nodules represent the actual transmission of the early lesions of human leprosy to monkeys, but grave doubts are thrown on this supposition by the fact that even dead leprosy bacilli are capable of producing similar nodules in certain animals.

The fact that only localized retrogressive lesions, and not the generalized infection, follow the inoculation of human leprosy material into monkeys has been considered to be due to the presence of a considerable amount of natural immunity in these animals against leprous infection. Attempts have therefore been made to break or reduce this natural immunity, and thereby produce the generalized disease in these animals. Oberdoerffer (1939) and Collier (1940) attempted to do this by feeding the monkeys on colocalia or by injecting them with sapotoxin prepared from colocalia. This sapotoxin is considered to lower the resistance by damaging the adrenals. Cochrane *et al.* (1937) tried to lower the defensive mechanism by removing the spleen of the monkeys prior to inoculating them with leprous material, a procedure previously used by other workers including Adler.

Oberdoerffer and Collier reported success in the transmission of human leprosy to monkeys which had been fed on colocalia or had received injections of the sapotoxin prepared from colocalia. The animals were inoculated twice by the insertion of a piece of nodule under the skin. In some of the inoculated monkeys lesions developed at the site of inoculation, in other parts of the skin, and on the face and the ears, and smears from these lesions and from the nasal mucosa showed acid-fast bacilli. This work has never been confirmed by any other worker. Moreover, the tissues of the monkeys, supposed to be suffering from generalized leprosy, were later examined histologically and bacteriologically in London, and the report was that the tissues showed no acid-fast bacilli and no evidence of leprous change.

Cochrane *et al.* (*loc. cit.*) obtained evidence of multiplication and spread of lepra bacilli in some of the monkeys in whom bits of lepromas were implanted in the abdomen after a preliminary splenectomy. One of their monkeys showed signs of generalized infection two months after it was inoculated with the human leprosy material. A small ulcer which proved to be a stitch abscess was noticed at the site of the operation wound two months after inoculation. Smears from the pus exuding from this abdominal wound showed many acid-fast bacilli including large and small globi. A week later small erythematous lesions, showing a tendency to ulcerate, were found on both sides of the original incision. The monkey looked ill, and was sacrificed. Smears from the abdominal wound showed clusters of acid-fast bacilli with many globi; scrapings from lesions of the abdominal wall also showed many bacilli; smears from liver and kidney also showed acid-fast bacilli. These workers concluded that it appeared possible to infect a monkey by performing preliminary splenectomy and embedding a nodule in the peritoneum preferably by fixing it to the splenic stump.

We have attempted to transmit human leprosy to splenectomized monkeys by a slightly modified method, and our results are reported in this paper.

PRESENT WORK.

The methods used.

Treatment of the animals previous to inoculation.—Six monkeys (*Macacus rhesus*) were employed in the experiment. Prior to being inoculated with lepromas material the animals were splenectomized under chloroform. An incision about 2" long was made in the abdominal wall over the region of the spleen. After retracting the skin, fascia and abdominal muscles, the spleen was located, and it was removed after ligating its pedicle securely with silkworm gut. The operation wound was then closed; the peritoneal and muscular layers were sutured with silkworm gut, and the skin was closed with Michel's clips. Due precaution was taken to prevent sepsis and concealed hæmorrhage. After the operation, and throughout the experimental period, the monkeys were housed under good hygienic conditions separate from other monkeys, as our previous experience had shown that the splenectomized monkeys are very susceptible to pulmonary tuberculosis.

The inoculum.—Clippings were obtained from the ear-lobes of an advanced case of leprosy of the lepromatous type. The cutaneous layer of the clippings was removed, and the remaining mass was ground with a glass-rod in a sterile hard-glass test-tube, containing a little ground-glass. The ground mass was then suspended in sterile normal saline, the suspension was allowed to stand for a few minutes, and the supernatant fluid was then pipetted off. A smear made from this fluid and stained by the Ziehl-Neelsen method showed abundant acid-fast bacilli and no contaminating organism. This emulsion was used for inoculating the monkeys.

Methods of inoculation.—Two weeks after the removal of the spleen, each monkey was inoculated intraperitoneally with 5 c.c. of the above emulsion.

The number of inoculations.—Each monkey was inoculated twice; the re-inoculation was made two months after the first inoculation, the same method being used.

Period of observation.—Three monkeys died between 3 and 3½ years after the first inoculation, and the remaining three were sacrificed 3½ years after the first inoculation.

The examinations made.

External examination.—During the period of observation, each monkey was periodically examined for the presence of any external signs of the disease, e.g. patches, nodules or ulcers, etc., on the face or body.

Lepromin test.—One year and four months after the second inoculation with leprous material, each monkey was tested intradermally with 0.02 mg. of nucleo-protein fraction of Hansen's bacillus prepared by the method previously described by one of us (Dharmendra, 1943). The test was repeated eight months later using the same antigen. Each monkey was observed for the appearance of any reaction at the site of injection.

Post-mortem examination.—After an animal died or was killed, it was first examined for macroscopic lesions in the various tissues, viz. liver, lungs, omentum, superficial and deep lymphatic glands. Smears were then made from the various tissues, stained by the Ziehl-Neelsen's method, and examined for acid-fast bacilli.

The findings.

External.—There was no external sign of leprous infection in any monkey. In some monkeys the tail appeared to be a little swollen and red about nine months after the first inoculation; this swelling and redness disappeared later, and was not considered to be of any significance.

Lepromin test.—Intradermal tests with nucleo-protein fraction of the Hansen's bacillus did not produce any reaction at the site of injection.

Post mortem.—In none of the animals were there found any gross pathological changes suggestive of leprous infection. The macroscopic and microscopic findings made in these animals are summarized below :—

Macroscopic.—In only one animal a post-operative peritoneal adhesion was found at the site of the removed spleen. There was no such local lesion in the peritoneum of the remaining animals.

In only one animal axillary and inguinal glands were slightly enlarged. No enlargement of any of the lymphatic glands was found in the remaining animals.

In only one animal a suspicious nodule was found in the omentum. No macroscopic lesions were found in any of the monkeys in the abdominal viscera and lungs.

Microscopic.—No acid-fast bacilli were found either in any of the lesions noted above or in any of the normal looking viscera and glands.

CONCLUSIONS.

1. It has not been possible to transmit human leprosy to splenectomized monkeys. Thus we have not been able to confirm the report of Cochrane *et al.* regarding the possibility of infecting splenectomized monkeys with human leprosy. However, our method of inoculation differed from that of Cochrane *et al.*; they introduced a piece of leprous nodule and fixed it to the splenic stump, while we injected leprous material intraperitoneally some days after splenectomy.

2. In none of our monkeys were acid-fast bacilli found at the post mortem. It is a well-recognized fact that the lepra bacilli, even the dead bacilli, have remarkable powers of persistence in tissues of living animals. The failure to find some of the injected bacilli in our monkeys is believed to be due to the long interval that elapsed between the inoculation and the post mortem. It appears that the injected bacilli do not persist for periods as long as three years or more.

3. The results of the lepromin test, performed with the nucleo-protein of the Hansen's bacillus, show that none of our monkeys had been sensitized to the products of this bacillus, even after being inoculated twice. It appears that the number of bacilli contained in the amount of suspension injected (10 c.c. in two injections) was not

sufficient to sensitize the animals; other workers have reported the development of an allergic state after repeated inoculations.

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THE EFFECT OF SULPHAPYRIDINE ON EXPERIMENTAL RAT LEPROSY.

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INTRODUCTION.

THE sulphanilamide group of drugs are known to possess a bacteriostatic or bactericidal action on a large variety of pathogenic organisms. A study was planned in the Leprosy Department, Calcutta School of Tropical Medicine, to investigate whether these drugs possess any such action on *Myco. lepræ muris*, an organism allied to the causative organism of human leprosy. The investigation was to include a study of the action of these drugs (a) *in vitro* and (b) *in vivo*.

The results of the first part of the study were reported by Dharmendra and Bose (1943) who concluded that sulphapyridine and sulphanilamide in a dilution of 1 in 1,000 possessed a bactericidal effect *in vitro* on *Myco. lepræ muris*, if allowed to act on the organism at 37°C. for 48 hours. The results of the second part of the study are reported here.

Some workers have studied the effect of these drugs on experimental tuberculosis in guinea-pigs, and on experimental rat leprosy in white rats, and have reported varying results. Rich and Follis (1938) and Greey *et al.* (1938) reported that these drugs had an inhibitory effect on the development of experimental tuberculosis in guinea-pigs, but Smithburn (1938), Kolmer *et al.* (1938) and Steinback and Dillon (1939) did not find them to have any such action. Cowdry and Ruangsiri (1941) found in a small series of leprosy rats that repeated subcutaneous injections of promin (sodium salt of p'p'-diaminodiphenyl sulphone-N₁N'-di dextrose sulfonate) did not modify the course of the disease in any way, but that the survival time of the treated animals was somewhat longer than that of the controls, and that at the conclusion of the experiment the treated animals were definitely in better condition than the controls. Krakower *et al.* (1943), on the other hand, found that in rats experimentally infected with rat leprosy, a diet containing 1 per cent of sulphanilamide given during the whole of the experimental period of one year, modified the course of the disease to a great extent. In the rats fed on sulphanilamide, only very small leprosy lesions developed, and the regional lymph glands were affected to a small extent without any other distant metastasis; the control animals developed large leprosy masses, local ulceration, and extensive metastasis in the glands and internal organs.

PRESENT INVESTIGATION.

The experimental procedure.

The drug used.—An aqueous suspension of sulphapyridine (M. & B. 693) was used. A 1 to 2 per cent suspension was made by grinding the sulphapyridine tablets in a mortar and adding the requisite amount of normal saline. For the administration of smaller doses a 1 per cent suspension was used, and for bigger doses a 2 per cent suspension was used, so that not more than 2.5 c.c. of the suspension had to be given to each rat at one time.

The method of administration.—The drug was given orally using an oesophageal cannula as described by Paul (1943). The cannula consists of a straight needle about 3 to 4 inches long, one end of which is made bulbous with lead, and the other end fits the nozzle of a syringe

tightly. The animal is held by an assistant with its head upwards and the jaw pushed back so that the head, neck and trunk are in a straight line. The operator then opens the mouth of the animal from the side, pressing on the tongue by means of an ordinary forceps. The bulbous end of the needle is introduced into the mouth and the needle is directed centrally and slightly backwards till it touches the back of the palate, it is then pushed very lightly downwards and goes easily down the pharynx into the oesophagus. Fluid is drawn inside the syringe to the required mark, and the rest of the syringe is filled with air. The syringe is now inverted and its nozzle is attached tightly to the proximal end of the needle. The fluid is then slowly injected. By this method a known amount of the drug is introduced directly into the stomach.

The dose.—In the preliminary experiment a daily dose of 40 mg. of the drug was given to white rats with an average weight of 80 g., but this dose was found to be too toxic and had later to be cut down. In a further experiment a small dose of 10 mg. was given and then gradually raised to 40 mg. The rats stood this increasing dose quite well.

The administration of the drug was started 2 or 3 days before the rats were experimentally infected with rat-leprosy material. The drug was given once a day in the afternoon, the animals being given their usual diet in the morning.

The methods of inoculation of rats.—An emulsion rich in rat leprosy bacilli was prepared from the liver and omentum of a rat suffering from experimental rat leprosy. One c.c. of this emulsion was given to each rat, 0.5 c.c. intraperitoneally into the abdomen, and 0.5 c.c. subcutaneously into the right groin.

The examinations made.—Each rat was observed daily at the time of feeding the drug. When an animal died or was sacrificed, its liver, spleen, omentum and superficial and deep lymphatic glands were examined macroscopically, and smears taken from these organs were stained by the Ziehl-Neelsen method and examined for the presence of acid-fast bacilli. No histological examination of the affected organs was made, since these organs had gross and characteristic lesions.

The preliminary experiment.

Details of the experiment.—Thirty-eight white rats with an average weight of 80 g. were used for this experiment. All the rats were infected with rat-leprosy material; 18 of these rats were fed with M. & B. 693, while the remaining 20 were not given any drug and served as controls.

The rats in the treated group received 40 mg. of M. & B. 693 daily for the first 3 days and 50 mg. daily for the next 3 days. Most of these rats could not tolerate this dose, and at the end of this period became seriously ill, looking very emaciated, with ulcers about the nostrils. The administration of the drug was suspended, but in spite of this 10 rats died within the next 10 days. The other 8 rats improved, and the administration of the drug was resumed after 2 weeks' suspension, but the dose was reduced to 10 mg. daily and later raised to 20 mg. daily. The treatment of these rats was continued for another 2½ months during which 3 rats received the full course of treatment amounting to a total of 1.16 g. of M. & B. 693; the other 5 died towards the end of the period of treatment having received a slightly lower total dose.

Results.—The 10 rats that received only a week's treatment and died within 3 weeks of the commencement of the experiment have been excluded from analysis since this period is too short; it was obvious that they died of the toxic effect of the drug. The other 8 rats died from 3 to 4½ months after the commencement of the experiment, and were thoroughly examined. In all of these 8 rats, lesions were found in the internal organs, and smears from the affected organs showed large number of acid-fast bacilli (*Mycob. lepræ muris*).

Of the 20 control rats (untreated) 2 died within 3 weeks of the inoculation and were not examined, the remaining 18 died from 2 to 6 months after the inoculation and all had developed lesions of rat leprosy.

The lesions in the rats in the two groups (the treated and the control) showed no differences either in nature or in extent.

The second experiment.

Details of the experiment.—Twenty-two white rats with an average weight of 98 g. were used. Twelve of these were fed on sulphapyridine and the remaining 10 were kept as control. The treated rats received 20 mg. of M. & B. 693 daily for 53 days, 30 mg. daily for 24 days and 40 mg. daily for 44 days. Three of the treated rats died within 3 weeks of the commencement of the treatment, while the other 9 received the treatment for 4 to 5 months. Three of these received a total dose of 3.27 g. of the drug and the other 6 slightly less.

Results.—Of the 12 treated rats 3 died within 2 weeks and therefore were not examined. The remaining 9 died or were sacrificed from 4 to 6 months after the commencement of the treatment. All showed heavy generalized infection with enlarged liver, spleen and omentum. A large number of acid-fast bacilli were found in smears from the affected organs. Of the 10 untreated rats one died too early to be examined, and the other 9 died from 3 to 4½ months after the inoculation. All these 9 rats showed heavy generalized infection with enlarged liver, spleen and omentum, and a large number of acid-fast bacilli were found in smears from the affected organs.

There was no difference in the two groups as regards the nature and extent of the lesions. Some of the treated rats survived for a longer period than any of the control rats; all the control rats died within 135 days after being inoculated, while 3 of the treated rats lived for 150 days or longer.

CONCLUSIONS.

1. It appears that rats with an average weight of 80 g. cannot tolerate orally an initial daily dose of 40 mg. to 50 mg. of sulphapyridine. However, after preliminary treatment with small repeated doses the animals can tolerate this dose quite well.

2. Under the conditions of the experiment described herein, sulphapyridine has failed to modify the course of experimental rat leprosy in white rats. The drug does not appear to have an inhibitory effect *in vivo* on *Myco. lepræ muris*, although *in vitro* it has been shown to have a bactericidal effect in a 1 in 1.000 dilution.

3. We have thus failed to confirm the work of Krakower *et al.* (*loc. cit.*) who reported that the feeding of sulphanilamide modified the course of the disease to a great extent, only small leprous lesions appearing in the treated rats, whereas large leprous lesions and extensive metastasis developed in the untreated control rats. Our findings are in keeping with the findings of Cowdry and Ruangsiri (*loc. cit.*), who found that repeated subcutaneous injections of promin did not modify the course of the disease in any way.

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A METHOD FOR THE DEMONSTRATION OF NEGRI BODIES.

BY

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STANDARD methods for the demonstration of Negri bodies, such as Mann's or Fotheringham's, are apt to be tedious and uncertain. Moreover, while they clearly differentiate the Negri body itself, staining is often uneven and unsuitable for a general histological study of the material, with special regard to encephalitis. The following method is technically simple and has given eminently satisfactory results :—

1. Fix thin slices of *hippocampus* and *cerebellum* in 10 per cent formal saline for 24 hours and prepare paraffin sections in the usual manner.

2. Take sections to tap-water.

3. Stain with Weigert's iron-hæmatoxylin for 10 minutes.

4. Rinse in water and transfer to Masson's ponceau 2R-acid fuchsin, diluted 1 : 10 with distilled water, for 5 minutes.

Stock.—Ponceau 2R	0·7 g.
Acid fuchsin	0·35 g.
Glacial acetic acid	1·0 c.c.
Aq. dest.	100·0 c.c.

5. Rinse in water and transfer to 1 per cent phosphomolybdic acid for 5 minutes.

6. If necessary, differentiate in 5·0 per cent sulphuric acid till sections are bluish red. Rinse well in water.

7. Dehydrate, clear and mount in the usual manner.

N.B.—(i) Stage 6 must be carefully controlled; 2 or 3 minutes usually is sufficient but the exact shade can only be learned by experience, (ii) use the $\frac{1}{6}$ " objective and artificial light, (iii) Negri bodies appear deep red, nuclei greyish black and cytoplasm pale mauve.

A PROTOZOAN PARASITE OF THE CENTRAL NERVOUS SYSTEM*.

BY

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I. INTRODUCTION.

THE findings to be described in this paper came to light in the course of an investigation undertaken primarily for the purpose of improving present methods for the diagnosis of rabies in animals.

Although it is generally accepted that the presence of Negri bodies in the brain is definite evidence of rabies infection, failure to demonstrate these bodies, while providing strong presumptive evidence against rabies, leaves the diagnosis in doubt and necessitates the issue of an inconclusive report.

In the course of these investigations the writer encountered appearances in the mid-brain of guinea-pigs, experimentally infected with rabies, which have not, apparently, been previously described. It is noteworthy that previous histological studies on the brains of rabid animals have been carried out chiefly on the hippocampus major and that the cerebellum, and particularly the mid-brain, have received little attention. The peculiar appearances encountered have been intensively investigated over the past six years and the conclusion has been reached that they are due to the presence of a parasite, probably protozoal in character, which is specific to the central nervous system. It has not been possible to identify this parasite with any previously described, such as *Encephalitozoon cuniculi*, or any known species of toxoplasma. A full description of this protozoal parasite is given in this paper. It has never been observed except in animals naturally or experimentally infected with rabies and the possibility of its association with the ætiology of rabies has, therefore, been discussed in some detail.

II. MATERIALS AND METHODS

1. *Strains of virus used.*

Most of the observations to be recorded were made on the brains of animals, chiefly guinea-pigs, experimentally infected with one or other of two strains of 'street' virus isolated from the brains of animals naturally infected with rabies. One of these strains was isolated from a jackal and the other from a dog. A more detailed description of each is given below :—

(a) *Jackal (J) strain.*—This strain was isolated from the brain of a rabid jackal in September 1937. The jackal's brain showed typical Negri bodies. A guinea-pig inoculated subdurally with a suspension of this jackal's brain developed 'furious' rabies on the fifteenth day after inoculation and died on the sixteenth day. Serial passage of this strain by subdural inoculation was continued in guinea-pigs during which the incubation period became progressively reduced. At the third passage (February 1939) the incubation period was twelve days: at the fifth passage (November 1939), ten days: and at the sixth passage (February 1940), eight days. From the seventh to the nineteenth passages, the incubation period varied from six to eight days, but from the twentieth passage onwards it was reduced to five days. Up to the time of writing this strain has been passaged serially in guinea-pigs twenty-five times. During the last nine passages, when the incubation period was reduced to only five days, Negri bodies were present only in very scanty numbers and were detectable only by prolonged examination.

* This work formed part of a thesis accepted for the D. Sc. degree in the Andhra University.

From the time of first isolation, over five hundred animals have been infected by various routes with this strain at various stages of its passage in guinea-pigs. Typical symptoms of rabies occurred in every case.

Guinea-pigs inoculated subdurally with the jackal strain showed symptoms of furious rabies during the earlier passages. They were very excitable and rushed about their cages, falling down from time to time. There was a marked tendency for the animals to bite the cage wires. In some cases death occurred suddenly during the excitable stage, while in others, paralysis was observed prior to death. From the twentieth passage onwards, when the incubation period was reduced to five days, death from paralytic rabies was the rule. In those animals in which infection was induced by intraperitoneal or intramuscular injection paralytic rabies developed.

(b) *Dog (D) strain*.—This strain was isolated from the brain of a rabid dog in August 1942. Typical Negri bodies were observed in the brain. A guinea-pig inoculated subdurally with a suspension of the brain of this animal developed paralysis on the eighth day and died on the ninth day. Negri bodies were found in the brain. Serial passage of this strain of virus was continued in guinea-pigs and, up to the time of writing, the strain has been passaged fifteen times. Up to the seventh passage the incubation period varied from six to eight days, but from the eighth passage onwards, it was reduced to only five days. All the guinea-pigs used for the passage of this strain showed symptoms of paralytic rabies. Typical Negri bodies were observed in the brains of animals used during the earlier passages, but from the eighth passage onwards, Negri bodies became increasingly reduced in number and size.

(c) *Other strains*.—Other strains of 'street' and 'fixed' virus were used in later experiments and will be described in the appropriate places in the text.

2. *Mode of infection of animals.*

Infection was induced by the inoculation of 0.2 c.c. of the clear supernatant after centrifuging a 10 per cent suspension of brain tissue. In most cases, experimental infections were induced by subdural inoculation. In some cases, however, animals were infected by the intramuscular (neck muscles), intravenous, intrasciatic or by the intraperitoneal route. Suspensions of other tissues, such as the sciatic nerve, or the submaxillary gland were used in some cases. In some of the later experiments, suspensions were filtered through Berkefeld V, N or W candles.

3. *Histological technique.*

Histological studies were carried out chiefly on tissues fixed in Zenker's fluid, embedded in paraffin, and stained with Mann's stain, iron-haematoxylin (long process) and, in most cases, with Giemsa's stain for twenty-four hours.

Serial sections of hippocampus major, cerebellum, and mid-brain at the level of the oculomotor nucleus, were made as a routine in all cases. Subsequent to the discovery of appearances suggestive of the presence of a protozoal parasite, sections of other organs were also studied, including the salivary gland, liver, spleen, kidney, lung and bone-marrow.

III. DESCRIPTION OF PROTOZOAL PARASITE.

The protozoal parasite to be described below was first encountered in the mid-brains of guinea-pigs infected with the jackal (J) strain described above and has been most exhaustively studied in material derived from them. Further observations were made on the brains of guinea-pigs infected with the dog (D) strain already described, as well as in experimentally infected rabbits and naturally infected jackals and dogs.

In all cases, the presence of the parasite was associated with typical Negri bodies, the latter being found regularly in the same sections and even in the same cells (Plate III, fig. 10).

The parasite to be described, with the exception of certain cystic forms of doubtful significance, invariably occurred in the cytoplasm of nerve cells, particularly those ventral to the central canal of the mid-brain. In a few cases, parasites were found also in the Purkinje cells of the cerebellum (Plate IV, fig. 15), and in the hippocampus major. Although exhaustive searches were made in sections and smears of other organs and tissues, no evidence of the parasite was detected except for some doubtful forms encountered in the ganglion cells of the salivary glands (Plate VI, fig. 46). The following description is based chiefly on a detailed study of the appearances observed in the brains of animals infected with the jackal (J) and the dog (D) strains referred to above.

'Spore' forms.—The smallest forms of the parasite encountered were minute 'spores'. They were regularly found in the mid-brains of the naturally infected dogs and jackals referred to later and in the brains of guinea-pigs inoculated with suspensions of their brains (Plate III, fig. 3). They were less frequently encountered in the brains of animals inoculated with the jackal (J) and dog (D) strains. The 'spores' stained dark blue with Giemsa's stain and blue with Mann's stain. They were always found in the cytoplasm of the nerve cells. Their sizes varied. The largest 'spores' were about half the size of *H. influenzae*, and the smallest could be seen only with difficulty under high magnification. The 'spores' might be found arranged singly (Plate III, fig. 1), or in pairs (Plate III, figs. 2 and 3), the latter arrangement being very common. They might occur in small groups (Plate III, fig. 4), or in large groups (Plate III, fig. 8). Sometimes, the cytoplasm of the nerve cell might be studded with these forms (Plate III, figs. 6 and 7). Occasionally, a group of 'spores' appeared to be surrounded by a thin wall (Plate III, fig. 5). The 'spores' might be found in the same cell with the other forms of the parasite (Plate VI, fig. 40).

Young forms.—The next stage of the parasite appeared to be the 'ring' form. The rings were variable in size. They might be very small, 0.5μ to 1μ (Plate III, figs. 9 to 11), or large, 7μ to 8μ (Plate IV, figs. 16 and 17). Intermediate sizes were also found (Plate III, fig. 12; Plate IV, figs. 13 to 15). The protoplasm of the parasite stained red with Giemsa's or Mann's stain, and was usually seen as a delicate, regular ring surrounding a vacuole. The chromatin mass stained blue with Mann's stain and dark blue with Giemsa's stain, and was usually single and prominent. It might be round or oval, and was variable in its position within the vacuole. It was usually situated in the centre of the vacuole (Plate III, fig. 12), but sometimes, it might lie close to the margin of the protoplasmic ring (Plate IV, fig. 15). The ring forms of the parasite were always found in the cytoplasm of nerve cells. A single ring might be present in a cell (Plate III, figs. 9 and 12; Plate IV, figs. 13, 14 and 16), or two rings (Plate III, fig. 11; Plate IV, fig. 15), or several rings (Plate III, fig. 10). Occasionally, the ring forms were found lying on the nuclear margin of the nerve cells (Plate IV, figs. 13 and 16).

Certain forms resembling the *accolé* forms of the malaria parasite were occasionally found. These forms consisted of a crescentic bridge of cytoplasm with a rounded chromatin dot. They might be in the cytoplasm of the nerve cell (Plate IV, fig. 19), or might be situated over the nuclear margin (Plate IV, figs. 18 and 20). The chromatin in these forms might be in the centre (Plate IV, figs. 18 and 19), or at one end (Plate IV, fig. 20).

Growing forms.—In slightly older forms, the chromatin mass was slightly larger, the protoplasm thickened and the parasite assumed a variety of shapes. It might be rounded (Plate IV, fig. 21), oval (Plate IV, figs. 22 to 24), fan-shaped (Plate V, fig. 25), pear-shaped (Plate V, fig. 26), elongated (Plate V, fig. 27), or irregular in outline (Plate V, fig. 28). The chromatin mass might be round or oval and situated in the centre or at the periphery of the protoplasm.

Dividing forms.—Segmentation of the chromatin appeared to be the next stage in the development of the parasite. In the segmenting forms the protoplasm appeared to be a solid mass with a varying number of chromatin dots. The chromatin might be divided into two fragments (Plate V, figs. 29 and 30), or three to six fragments (Plate V, figs. 31 to 34), or more numerous fragments (Plate V, fig. 35; Plate VI, fig. 37). The fragments of divided chromatin might be arranged in linear fashion (Plate V, figs. 32 and 33), or in a cluster

(Plate V, figs. 34 and 35). The mode of arrangement seemed to depend on the nature of the space available within the cytoplasm of the cell. The nucleus of the nerve cell might be pushed to one side by the dividing form (Plate V, fig. 35). In certain cases, the segmenting forms appeared to be surrounded either partly or completely by a thin wall.

The condensation of the cytoplasm round the fragments of chromatin (Plate VI, fig. 37) and separation of oval-shaped bodies with a single nucleus resembling merozoites (Plate VI, fig. 38) were observed.

The limits of toleration of the nerve cell to these bodies growing within its cytoplasm were surprising. The whole cell might be filled with the dividing form and the nucleus and the nucleolus might still remain intact (Plate V, fig. 36). Degenerative changes were generally more marked in the nerve cells showing the 'spore' forms compared with those showing the other stages of the parasite.

Finally, the cell might disintegrate and forms resembling merozoites set free (Plate VI, fig. 39).

It was possible to demonstrate all the stages of development of the parasite in the brain of a single animal.

During this study, certain 'cystic' forms were encountered in the brains of four guinea-pigs. The relationship, if any, of these forms to the parasite is not clear. These rounded cysts appeared to represent a collection of spores enclosed within a wall. The cysts were situated outside the nerve cells in all cases. They were encountered in the hippocampus major, cerebellum and mid-brain of three different guinea-pigs inoculated intramuscularly with the jackal (J) strain. They were also found in the mid-brain of a guinea-pig inoculated subdurally with the dog (D) strain. Individual spores forming a cyst appeared to be uniform in size but the size of the spores in different guinea-pigs varied. Whether the larger spores represented the mature forms of the smaller ones, it is difficult to say.

The *Feulgen reaction* for the demonstration of chromatin was tried on sections of the brains of animals showing the parasite. The brains were fixed in Zenker's fluid. It was found that the chromatin of the parasite, generally, did not give the Feulgen reaction.

The changes in the various constituents of the nerve cells in the brains showing the presence of the parasite were studied in some cases. No evidence was found to suggest that the various forms of the parasite were the result of alterations in the constituents of nerve cells.

IV. CONDITIONS UNDER WHICH THE PARASITE WAS OBSERVED.

No evidence of the parasite described above was detected in the brains (hippocampus major, cerebellum and mid-brain) of sixty guinea-pigs which died as a result of infection with the jackal (J) strain of virus prior to the sixth serial passage in guinea-pigs, i.e. when the incubation period varied from ten to fifteen days. Negri bodies were, however, invariably present. Subsequent to the sixth passage with this strain, i.e. when the incubation period of the virus was reduced to eight days, the parasite was invariably found. Several attempts were made to detect the presence of the parasite in the brains of guinea-pigs inoculated with other strains of 'street' virus without success. In each case the incubation period in guinea-pigs was fifteen days or more. When, however, the dog (D) strain described above was isolated the incubation period on first passage was only eight days and forms identical with those described above were demonstrable in the brain. The parasite was detected in the brains of thirty-eight guinea-pigs inoculated subdurally with the jackal (J) strain and twenty guinea-pigs inoculated subdurally with the dog (D) strain. The brains of one hundred and forty-two guinea-pigs inoculated intramuscularly with the jackal (J) strain and ten guinea-pigs inoculated intramuscularly with the dog (D) strain, were carefully examined and the parasite was detected in all of these animals. The brains of two guinea-pigs inoculated intraperitoneally with the jackal (J) strain and two guinea-pigs inoculated intraperitoneally with the dog (D) strain, revealed the presence of the parasite in each

case. The parasite was demonstrable in the brain of a guinea-pig whose sciatic nerve was aseptically exposed and pricked by a needle infected with the jackal (J) strain virus. The occurrence of the parasite in the brains of the experimental animals was, therefore, independent of the mode of infection of the experimental animals used.

The clear supernatant obtained after centrifugalization of a 10 per cent suspension of the brain of guinea-pigs infected with the jackal (J) strain or dog (D) strain virus in serum water was filtered through Berkefeld V, N and W candles and the filtrates inoculated subdurally into guinea-pigs. The brains of two guinea-pigs inoculated with the V filtrate (Plate IV, fig. 13; Plate V, fig. 33) and one guinea-pig inoculated with the N filtrate (Plate IV, fig. 17) of the jackal (J) strain virus showed the presence of the parasite. The parasite was also demonstrable in the brain of a guinea-pig inoculated with the V filtrate and another inoculated with the N filtrate of the dog (D) strain virus. The Berkefeld W filtrates of the jackal (J) or dog (D) strains of virus did not prove to be infective.

The parasite was detected in the brains of guinea-pigs inoculated subdurally with suspensions of the submaxillary glands of other guinea-pigs which died as a result of infection with the jackal (J) or dog (D) strain of virus (Plate III, fig. 5; Plate IV, fig. 14).

In one guinea-pig inoculated with the jackal (J) strain and killed at the onset of the disease the parasite was found in large numbers in the mid-brain but the Negri bodies were very scanty. Relatively smaller numbers of parasites and numerous Negri bodies were encountered in the brain of another guinea-pig inoculated with the same material but allowed to die of the disease.

The parasite was also demonstrable in the brains of other animals inoculated with the jackal (J) and dog (D) strain.

Rabbits.—The brains of two rabbits inoculated subdurally with the jackal (J) strain and two rabbits inoculated subdurally with the dog (D) strain showed the presence of the parasite. The parasite was also demonstrated in the brains of guinea-pigs inoculated with suspensions of the brains (Plate IV, fig. 24; Plate V, fig. 36), or sciatic nerves (Plate III, fig. 12) of rabbits infected with the jackal (J) or dog (D) strain. The brain of a rabbit inoculated intraperitoneally with the jackal (J) strain and the brains of two rabbits inoculated intraperitoneally with the dog (D) strain revealed the presence of the parasite in every case. The parasite was demonstrable in the brain of a rabbit inoculated intravenously with the jackal (J) strain and in another rabbit inoculated intravenously with the dog (D) strain. The parasite was not detectable in the brains of twenty-five rabbits inoculated intramuscularly with the seventh guinea-pig passage of the jackal (J) strain. It was, however, encountered in the brains of six rabbits inoculated intramuscularly with the tenth guinea-pig passage of the jackal (J) strain virus. The brain of a rabbit inoculated intrasciatically with the dog (D) strain showed the presence of the parasite.

The incidence and morphology of the parasite in rabbits were similar to those observed in guinea-pigs. The parasite was never encountered in the liver, spleen, kidneys, lungs, bone-marrow and blood of any of the experimental animals examined.

Dogs.—A few small Negri bodies were detectable in the brain of a dog inoculated intramuscularly with the jackal (J) strain and another dog inoculated intramuscularly with the dog (D) strain virus but no evidence of the parasite was observed in either of these animals. The parasite was, however, demonstrable in the brains of guinea-pigs inoculated with the brains of the dogs.

Forms resembling the parasite in natural rabies.—The possibility of the parasite being a natural infection common to dogs and jackals, unconnected with rabies infection, was investigated. The brains of apparently healthy dogs as well as those which had died of infections other than rabies were carefully examined for the occurrence of the parasite. The brains of two jackals which had been killed because they had strayed into inhabited localities were also studied. The parasite was never encountered in their brains, nor were Negri bodies found.

TABLE.

RESULT OF EXAMINATION OF THE
BRAIN OF THE NATURALLY
INFECTED ANIMAL.

RESULT OF INOCULATING 0.2 C.C. OF THE CLEAR SUPERNATANT FROM
A 10 PER CENT SUSPENSION OF THE BRAIN SUBDURALLY
INTO GUINEA-PIGS.

Animal.	NORM. BODIES.			Incubation period in days.	Symptoms.	NORM. BODIES.			PARASITES.		
	Hippocampus.	Cerebellum.	Mid-brain.			Hippocampus.	Cerebellum.	Mid-brain.	Hippocampus.	Cerebellum.	Mid-brain.
1 Dog	++	++	++	2	Furious rabies	+	+	+	+	+	+
2 "	++	++	++	2	"	+	+	+	+	+	+
3 "	++	++	++	2	"	+	+	+	+	+	+
4 "	++	++	++	2	"	+	+	+	+	+	+
5 "	++	++	++	2	"	+	+	+	+	+	+
6 "	++	++	++	2	"	+	+	+	+	+	+
7 "	++	++	++	2	"	+	+	+	+	+	+
8 "	++	++	++	2	"	+	+	+	+	+	+
9 "	++	++	++	2	"	+	+	+	+	+	+
10 "	++	++	++	13	"	+	+	+	+	+	+
11 Jackal	++	++	++	6	Paralytic rabies	+	+	+	+	+	+
12 "	++	++	++	8	Furious rabies	+	+	+	+	+	+
13 "	++	++	++	6	Paralytic rabies	+	+	+	+	+	+
14 "	++	++	++	7	Paralytic rabies	+	+	+	+	+	+
15 "	++	++	++	6	Furious rabies	+	+	+	+	+	+
16 "	++	++	++	2	"	+	+	+	+	+	+
17 "	++	++	++	8	"	+	+	+	+	+	+
18 "	++	++	++	13	"	+	+	+	+	+	+
19 "	++	++	++	8	"	+	+	+	+	+	+
20 Calf	++	++	++	2	"	+	+	+	+	+	+

The brains of one hundred dogs, which died of natural rabies, were studied. These brains were sent to the Institute for examination from outside areas. The hippocampus major and the cerebellum alone were available for examination in eighty-eight cases, while the hippocampus major, the cerebellum, and the mid-brain at the level of the oculomotor nucleus were available for study in only twelve cases. Negri bodies were found in the brains of all these animals. Forms resembling the ring forms and the schizonts of the parasite described above were encountered in the hippocampus major in one case (Plate VI, fig. 41) and in the mid-brain in another.

A recent epidemic of rabies among the canine population around Coonoor provided a splendid opportunity to investigate the possible occurrence of the parasite in naturally infected animals. So far, it has been possible to isolate twenty strains of rabies virus behaving like the jackal (J) and dog (D) strains. Of these ten were from dogs, nine from jackals and one from a cow-calf. Particulars regarding these strains are summarized in the Table.

Various forms of the parasite, a description of which is given below, were encountered in the mid-brains of these dogs, jackals and calf. They were invariably found in the cytoplasm of nerve cells, particularly those ventral to the central canal of the mid-brain. The parasite was not demonstrable in the liver, spleen, kidneys, lungs, bone-marrow or blood of any of these animals.

The 'spore' forms of the parasite, already described, were found regularly in the mid-brains of all the dogs, jackals and calf (Plate III, figs. 1 to 4 and 6 to 8). The 'spores' were encountered in larger numbers in the brains of jackals than of dogs and particularly among jackals in which the incubation period was relatively short.

Ring forms (Plate III, fig. 9) and growing forms (Plate V, fig. 28) were found only in small numbers. The cytoplasm of the parasite stained red and the chromatin dark blue with Giemsa's stain.

Forms resembling the schizonts of the parasite were commonly found in the mid-brains of the dogs, jackals and calf (Plate VI, figs. 40 and 42 to 45). These forms were variable in size. They might be found lying on the nuclear membrane (Plate VI, figs. 42 and 43). Occasionally, the 'spore' forms and the schizonts were encountered in the same cell (Plate VI, fig. 40). In some cases, a thin wall, which seemed to be formed by the cytoplasm of the cell, was found to surround the schizont-like form either partly (Plate VI, figs. 43 and 44) or completely (Plate VI, fig. 45). The appearances of the latter forms were not different from those of Negri bodies.

The incubation period in guinea-pigs inoculated subdurally with the virus from the dogs, jackals and calf generally varied from six to eight days (*vide* Table). In two instances it was thirteen days, but in both these cases the incubation period in the subsequent passage was only six days. The incubation period was generally short (six days) in guinea-pigs inoculated with the brains of jackals showing a predominance of the 'spore' forms. Guinea-pigs usually showed signs of furious rabies, only those inoculated with the brains of jackals 5 and 18 dying of paralytic rabies. The parasite was encountered in the brains of guinea-pigs inoculated with the brains of the dogs, jackals and calf. The incidence and morphology of the parasite were identical with those observed in the brains of guinea-pigs inoculated with the jackal (J) and dog (D) strains.

Berkefeld V and N filtrates of the brains of some of the dogs and jackals included in the Table were inoculated subdurally or intramuscularly into guinea-pigs. Filtration through Berkefeld W candle was not tried because the results with the jackal (J) and dog (D) strains of virus had been negative. The parasite was demonstrable in the brains of guinea-pigs inoculated with the Berkefeld V filtrates of two dogs (297 and 344) and four jackals (15, 18, 298 and 359) and the Berkefeld N filtrate of one dog (342).

V. THE NATURE OF THE PARASITE.

Having arrived at the conclusion that the appearances described above represented various stages in the life-cycle of a protozoan parasite, it was suspected that this parasite

Note.—All microphotographs are taken from sections of mid-brains unless otherwise stated.

EXPLANATION OF PLATE III.

- Fig. 1. Nerve cell showing single spores scattered in the cytoplasm. Giemsa. $\times 840$.
„ 2. Nerve cell showing spores scattered in the cytoplasm. Some of them are in groups of two. Giemsa. $\times 810$.
„ 3. Nerve cell showing spores in groups of two. Giemsa. $\times 810$.
„ 4. Nerve cell showing spores arranged in small groups in the cytoplasm. Giemsa. $\times 810$.
„ 5. Nerve cell showing a collection of spores which appears to be surrounded by a thin wall. Giemsa. $\times 700$.
„ 6. Nerve cell showing a collection of small spores. Giemsa. $\times 810$.
„ 7. Figure showing two nerve cells studied with spore forms. Giemsa. $\times 840$.
„ 8. Nerve cell showing a group of large spores. Giemsa. $\times 810$.
„ 9. Nerve cell showing a small ring. Giemsa. $\times 810$.
„ 10. Nerve cell showing several small rings and a typical Negri body. Mann. $\times 700$.
„ 11. Nerve cells showing rings of different sizes. Mann. $\times 700$.
„ 12. Nerve cell showing a small ring with a central chromatin. Mann. $\times 700$.

PLATE III.

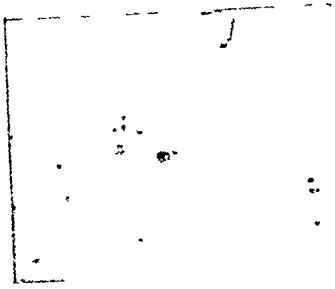


Fig. 1.



Fig. 2.



Fig. 3.



Fig. 4.



Fig. 5.

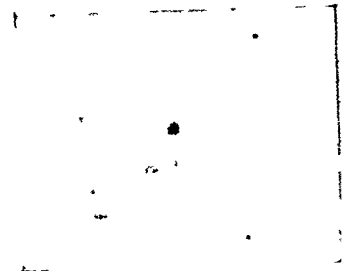


Fig. 6.

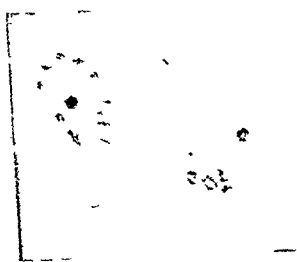


Fig. 7.



Fig. 8.

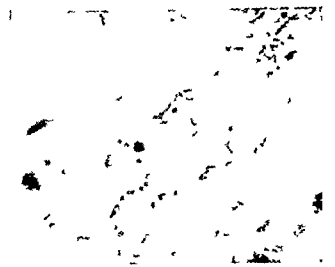


Fig. 9.

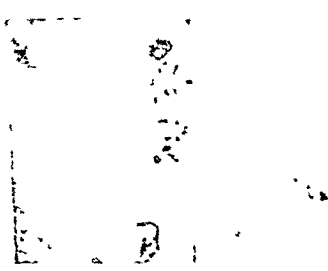


Fig. 10.



Fig. 11.



Fig. 12.

PLATE IV.

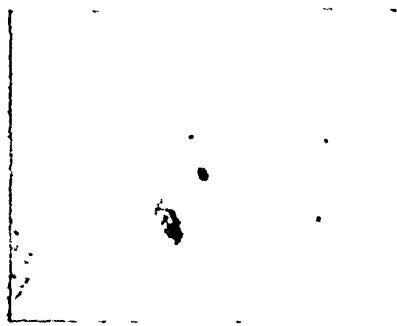


Fig. 13.



Fig. 14.



Fig. 15.



Fig. 16.



Fig. 17.



Fig. 18.



Fig. 19.



Fig. 20.



Fig. 21.



g. 22.



Fig. 23.

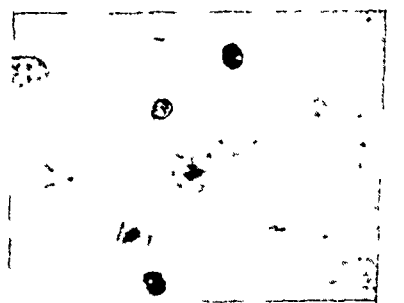


Fig. 24.

Note.—All microphotographs are taken from sections of mid-brains unless otherwise stated.

EXPLANATION OF PLATE IV.

- Fig. 13. Nerve cell showing a ring lying on the nuclear margin. Giemsa. $\times 700$.
„ 14. Nerve cell showing a ring. Giemsa. $\times 700$.
„ 15. A Purkinje cell of the cerebellum showing a circular and oval ring. Mann. $\times 700$.
„ 16. Nerve cell showing a large ring from lying on nuclear margin. Giemsa. $\times 700$.
„ 17. Nerve cell showing a large ring and two smaller ones. Mann. $\times 700$.
„ 18. Nerve cell showing an *accolé*-like form lying on nuclear margin and a growing form. Giemsa. $\times 700$.
„ 19. Nerve cell showing an *accolé*-like form with central chromatin lying in the cytoplasm. Mann. $\times 700$.
„ 20. Nerve cell showing an *accolé*-like form with chromatin at one end and lying on nuclear margin. Mann. $\times 700$.
„ 21. Nerve cell showing a rounded growing form. Mann. $\times 700$.
„ 22. Nerve cell showing a growing form lying on nuclear margin. Mann. $\times 700$.
„ 23. Nerve cell showing two growing forms, one lying on nuclear margin and the other in the cytoplasm of the cell. Mann. $\times 700$.
„ 24. Nerve cell showing a growing form. Mann. $\times 700$.

Note.—All microphotographs are taken from sections of mid-brains unless otherwise stated.

EXPLANATION OF PLATE V.

- Fig. 25. Nerve cell showing a fan-shaped growing form with chromatin at the periphery. Mann. $\times 700$.
- „ 26. Nerve cell showing a pear-shaped growing form with chromatin at the thicker end. Giemsa. $\times 700$.
- „ 27. Nerve cell showing an elongated growing form with chromatin in the centre. Mann. $\times 700$.
- „ 28. Nerve cell showing an irregular growing form. Giemsa. $\times 840$.
- „ 29. Nerve cell showing a dividing form lying on nuclear margin. The chromatin is divided into two fragments. Giemsa. $\times 700$.
- „ 30. Nerve cell showing a dividing form with chromatin divided into two fragments. Mann. $\times 700$.
- „ 31. Nerve cell showing a dividing form with three fragments of chromatin. Mann. $\times 700$.
- Figs. 32 and 33. Nerve cell showing a dividing form with fragments of chromatin arranged in a linear fashion. Giemsa. $\times 700$.
- Fig. 34. Nerve cell showing a dividing form with five chromatin dots lying on nuclear margin. Giemsa. $\times 700$.
- „ 35. Nerve cell showing a dividing form with several fragments of chromatin arranged in a group. The nucleus of the nerve cell is pushed to one side. Giemsa. $\times 700$.
- „ 36. Nerve cell showing three schizont-like forms occupying major portion of the cytoplasm of the cell. Mann. $\times 700$.

PLATE V.

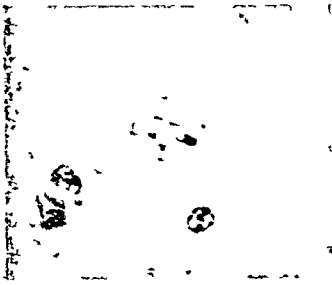


Fig. 25.

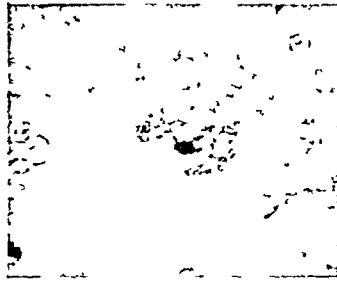


Fig. 26.



Fig. 27.

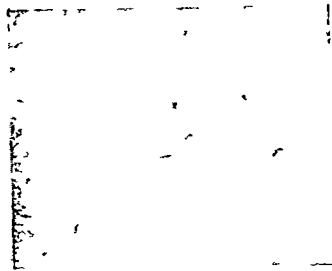


Fig. 28.



Fig. 29.



Fig. 30.



Fig. 31.

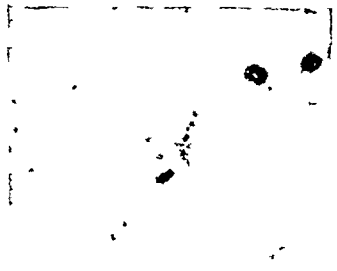


Fig. 32.



Fig. 33.

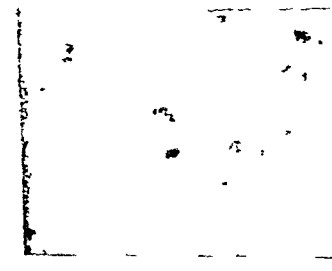


Fig. 34.



Fig. 35.



Fig. 36.

Note.—All microphotographs are taken from sections of mid-brains unless otherwise stated.

EXPLANATION OF PLATE V.

- Fig. 25. Nerve cell showing a fan-shaped growing form with chromatin at the periphery. Mann. $\times 700$.
- „ 26. Nerve cell showing a pear-shaped growing form with chromatin at the thicker end. Giemsa. $\times 700$.
- „ 27. Nerve cell showing an elongated growing form with chromatin in the centre. Mann. $\times 700$.
- „ 28. Nerve cell showing an irregular growing form. Giemsa. $\times 840$.
- „ 29. Nerve cell showing a dividing form lying on nuclear margin. The chromatin is divided into two fragments. Giemsa. $\times 700$.
- „ 30. Nerve cell showing a dividing form with chromatin divided into two fragments. Mann. $\times 700$.
- „ 31. Nerve cell showing a dividing form with three fragments of chromatin. Mann. $\times 700$.
- Figs. 32 and 33. Nerve cell showing a dividing form with fragments of chromatin arranged in a linear fashion. Giemsa. $\times 700$.
- Fig. 34. Nerve cell showing a dividing form with five chromatin dots lying on nuclear margin. Giemsa. $\times 700$.
- „ 35. Nerve cell showing a dividing form with several fragments of chromatin arranged in a group. The nucleus of the nerve cell is pushed to one side. Giemsa. $\times 700$.
- „ 36. Nerve cell showing three schizont-like forms occupying major portion of the cytoplasm of the cell. Mann. $\times 700$.

Note.—All microphotographs are taken from sections of mid-brains unless otherwise stated.

EXPLANATION OF PLATE VI.

- Fig. 37. Nerve cell showing a form resembling a mature schizont. Giemsa. $\times 700$.
- „ 38. Nerve cell showing oval-shaped bodies, each with a single chromatin resembling merozoites. Giemsa. $\times 700$.
- „ 39. Figure showing disintegration of nerve cell and liberation of merozoite-like forms. Mann. $\times 700$.
- „ 40. Figure showing three nerve cells. The middle cell contains spores and two schizont-like forms. The other two cells contain spores. Giemsa. $\times 840$.
- „ 41. Nerve cell from the hippocampus of a dog showing a schizont-like form. Iron-haematoxylin. $\times 700$.
- „ 42. Nerve cell showing a schizont-like form lying on nuclear margin. Appearances suggest that a wall round the schizont is being formed. Giemsa. $\times 700$.
- „ 43. Nerve cell showing a schizont-like form lying on nuclear margin partly surrounded by a wall. Giemsa. $\times 840$.
- „ 44. Nerve cell showing a schizont-like form surrounded by a definite wall. Giemsa. $\times 840$.
- „ 45. Nerve cell showing a schizont-like form completely surrounded by a wall. Giemsa. $\times 840$.
- „ 46. Figure shows two ganglion cells of the submaxillary gland. The cell at the top shows a form resembling a ring. Mann. $\times 700$.
- „ 47. A cyst containing small spores situated outside the nerve cell in the mid-brain. Mann. $\times 700$.
- „ 48. A large cyst containing spores situated outside the nerve cell in the mid-brain. Mann. $\times 700$.

might be a natural infection either in the animals from which the rabies virus strains were originally derived, or a natural infection in the experimental animals used which was being passed along with rabies virus. A search of the literature revealed no previous record of infection with any parasite the description of which corresponds to that reported in this paper. There is, however, a general resemblance to *Encephalitozoon cuniculi* and to certain species of toxoplasma. The evidence against a coincident infection of this kind is summarized below :—

1. *A natural infection distinct from rabies in dogs and jackals.*—A careful examination of the brains of some apparently healthy dogs as well as those which had died of infections other than rabies did not reveal the presence of the parasite, nor was it encountered in the brains of two jackals which showed no evidence of rabies infection.

2. *A natural infection in laboratory animals used.*

(a) *Encephalitozoon cuniculi.*—The cystic lesions found outside the nerve cells in the brains of guinea-pigs inoculated with the jackal (J) and dog (D) strains of virus bear a resemblance to those found in *E. cuniculi* infection. The chief reasons against a coincident infection with the above parasite are :—

- (i) The cysts were found in the brains of only four guinea-pigs out of the several hundred brains examined. They were never encountered in the brains of dogs and jackals showing the presence of the parasite or in the brains of guinea-pigs inoculated with suspensions of their brains. It is, therefore, difficult to say whether these cysts have any relation to the parasite in question.
- (ii) *E. cuniculi* infection, as far as the author is aware, has not been described in guinea-pigs so far.
- (iii) Although rabbits are very susceptible to *E. cuniculi* infection, the characteristic appearances of this disease were never found in the brains of rabbits inoculated subdurally, intramuscularly, intravenously and intrasciatically with the jackal (J) and dog (D) strain of virus passaged in guinea-pigs.
- (iv) The various stages of the parasite reported in the paper have not been described in *E. cuniculi* infection.
- (v) In *E. cuniculi* infection the parasite can be demonstrated in liver, spleen and kidney but careful examination has not revealed the presence of the parasite described in any of these organs.
- (vi) Symptoms associated with *E. cuniculi* infection (da Fano, 1924) were never observed in any of the guinea-pigs or rabbits inoculated with suspensions of the brains showing the parasite. The symptoms observed were always those of rabies.

(b) *Toxoplasma.*—The following evidence is against the possibility of a coincident infection with toxoplasma :—

- (i) In toxoplasma infection of guinea-pigs and rabbits, the parasite has not been described in the brain.
- (ii) The post-mortem appearances in guinea-pigs and rabbits inoculated with the parasitic material did not show the characteristic lesions described in toxoplasma infection.
- (iii) Smears and sections of liver, spleen, kidney, lung and bone-marrow of our experimental animals showed no evidence of parasites of the toxoplasma or other type.

3. *A latent natural infection in laboratory animals excited by superinfection with rabies.*—The following observations indicate that the possibility of an unknown latent infection in the experimental animals, which was activated by rabies infection, is very unlikely :—

- (i) Several guinea-pigs and rabbits caught at random from the runs were killed and their brains and other organs examined. The parasite was never found.

- (ii) Guinea-pigs and rabbits selected from the same runs, which died as a result of subdural infection with Paris strain of rabies 'fixed' virus, did not show the presence of the parasite.
- (iii) The parasite was not encountered in the brains of guinea-pigs inoculated with strains of 'street' virus whose incubation period was fifteen days or more.
- (iv) The demonstration of forms resembling the parasite in the brains of rabid dogs and jackals infected in nature renders the possibility of a latent infection in experimental animals unlikely.

VI. THE RELATIONSHIP OF THE PROTOZOAL PARASITE DESCRIBED TO RABIES.

The appearances described above have been observed only in association with rabies infection. While the writer is fully aware of the possibility that the protozoal parasite described may be a coincident infection in no way connected with rabies infection, he has been unable to demonstrate this by any of the experimental procedures employed. On the other hand, such evidence as is available suggests that the protozoal parasite described is not unconnected with the ætiological agent of rabies.

The observations with the jackal (J) and dog (D) strains of virus indicate that the parasite is demonstrable in the brains of animals inoculated with the virus at a certain stage of passage either in nature or in the laboratory, i.e. when the incubation period of the virus given subdurally into guinea-pigs varies from six to eight days. The finding, that the incubation period is the same in guinea-pigs inoculated subdurally with suspensions of the brains of naturally infected dogs and jackals showing the presence of the parasite, supports this view. The parasite is seldom found when the incubation period of the virus by repeated passage in guinea-pigs becomes 'fixed'.

The occurrence of the parasite in the brains of experimental animals inoculated with the jackal (J) or dog (D) strain of virus was independent of the mode of infection. The parasite, with the exception of the cystic lesions already referred to, was generally found in the cytoplasm of nerve cells, particularly those ventral to the central canal of the mid-brain. It was not demonstrable in the liver, spleen, kidney, lung, blood or bone-marrow whatever mode of infection was adopted. The parasite was found in the mid-brain of ten dogs, nine jackals and one calf infected in nature and in the brains of guinea-pigs inoculated with a suspension of the brain of each of these animals. The parasite was not encountered in sections and smears of other organs and tissues of the dogs, jackals and guinea-pigs. These findings indicate that the parasite is specific to the central nervous system.

The following observations suggest that the parasite is ætiologically related to the Negri body: (i) Negri bodies were found with the parasite in the same section or in the same cell. (ii) Negri bodies were regularly encountered in the brains of animals showing the presence of the parasite. (iii) When the virus, by repeated passage in guinea-pigs, become 'fixed' and Negri bodies become scanty, the parasite was also difficult to find. (iv) In the brains of animals killed at the onset of the disease the parasite was found in large numbers but Negri bodies were very scanty. Relatively small numbers of parasites and numerous Negri bodies were encountered in the brains of animals allowed to die of the disease. These observations suggest that Negri bodies may represent a later stage in the development of the parasite. (v) Negri bodies are eosinophilic while the granules they contain are basophilic. The 'spore' forms and the chromatin of the other stages of the parasite are basophilic like the chromatin of the nerve cell, while the cytoplasm of the parasite is eosinophilic. Thus, the internal granules of the Negri body resemble the chromatin of the parasite in their staining reaction, while the rest of the Negri body stains like the cytoplasm of the parasite. This observation is particularly interesting as in some protozoa, such as the malarial parasite, the chromatin is eosinophilic and the cytoplasm basophilic. (vi) Negri bodies, like the parasite, generally do not give the Feulgen reaction for chromatin. (vii) Observations on naturally infected dogs and jackals and on guinea-pigs inoculated with their brains suggest that Negri bodies

may represent the schizont form of the parasite ensheathed by an envelope, which, possibly, may be formed from the cytoplasm of the cell.

The experimental evidence with the jackal (J) and dog (D) strains of virus shows that the parasite can be demonstrated in the brains of animals inoculated with : (i) suspensions of the brains of guinea-pigs, rabbits and dogs which died of the disease, (ii) suspensions of the peripheral nerves (sciatic nerves) of animals which died of the disease, (iii) suspensions of the salivary glands of animals which died of the disease, (iv) Berkefeld V and N filtrates of the brains of animals which died of the disease, and (v) suspensions of the brains of animals inoculated intrasciatically. The experiments with the other strains of virus isolated from naturally infected dogs and jackals also confirm these findings. The parasite can be demonstrated in the brains of guinea-pigs inoculated with : (i) suspensions of the brains of dogs and jackals, and (ii) Berkefeld V and N filtrates of their brains. The above results are in conformity with the accepted findings in rabies.

The main arguments against the theory that the aetiological agent in rabies is a protozoon are : (i) the developmental cycles described by various workers are based on very inadequate grounds, and (ii) so far as is known, no protozoa exist which have, as part of their life-cycle, invisible filtrable elements.

The observations recorded in this paper show that it is possible to demonstrate, in the brains of animals infected with natural or experimental rabies, a parasite whose morphological appearances and method of multiplication appear to be those of a protozoon.

It is well known that rabies virus can pass through only the coarser bacterial filters. Glusmann *et al.* (1930) studied the degree of filtrability of rabies virus exhaustively and came to the conclusion that it was not a true filter-passer and that in certain stages it was a visible microbe. The 'spore' forms described in the paper are much smaller than *H. influenza* which can pass through Berkefeld V candle at high pressure (Dible, 1932). Some of these forms are just visible under high magnification and it is possible that invisible forms also exist. Therefore, it should be possible for these forms to pass through the coarser candles such as Berkefeld V and N. The observation that the 'spore' forms can be demonstrated with the other stages of the parasite in the brains of animals inoculated with Berkefeld V and N filtrates suggest that the 'spores' may represent the filtrable phase of the parasite. The demonstration of the 'spores' and the other stages of the parasite in a single cell lends support to this view.

Levaditi, Nicolau and Schoen (1924, 1924a, 1924b, 1924c) put forward the theory that rabies virus was a microsporidium which had a definite life-cycle with two phases. The first one was a filtrable phase in which the agent resembled spores. The spores passed along the nerves from the site of the wound and reached the central nervous system. There they underwent intracellular proliferation to form pansporoblasts or Negri bodies. The pansporoblasts which represented the visible phase of the organism contained 'plages' each of which was an aggregation of microsporidium spores. Round them the neurone secreted a hyaline substance which formed a capsule, isolating the colony from the rest of the cell. The development of the pansporoblast depended on the virus and the host. If the virus was very virulent it might destroy the nerve cell before the pansporoblast could form. The absence of Negri bodies in 'fixed' virus infections might be explained by the fact that the rate of multiplication of the ultra-microscopic forms is so rapid that the animal dies before the cystic forms are produced. According to the same authors only one phase of the Negri body (pansporoblast) is visible, the spore phase of the organism being invisible and filtrable. There is little or no evidence in support of this plausible theory.

The evidence presented in this paper suggests that the protozoan parasite described might be the aetiological agent in rabies, the 'spore' forms representing the filtrable phase. There seem to be two methods of multiplication of the parasite. Firstly, the 'spores' may undergo intracellular proliferation by repeated division, this possibly being the chief method of multiplication in 'fixed' virus infections. The difficulty of demonstrating Negri bodies and the other stages of the parasite in infections with the Paris strain of rabies virus could

be explained by assuming that only the invisible 'spores' capable of passing through candles like Berkefeld N (which prevent the passage of all bacteria) are present. Secondly, the 'spores' may develop into schizonts. From these, forms resembling merozoites are formed and these are set free as a result of the disintegration of the nerve cell. These forms may invade new cells. The Negri body itself may be a mature form of the parasite round which an envelope has been formed from the cytoplasm of the cell.

SUMMARY.

1. A parasite, apparently protozoal in character, originally found in the mid-brains of guinea-pigs experimentally infected with rabies 'street' virus has been studied and a full description of its morphology has been given.

2. An account has been given of the occurrence and behaviour of this protozoal parasite in other animals.

3. Appearances, similar to the various stages in the life-cycle of the parasite described, have been observed in the mid-brain of dogs and jackals suffering from natural rabies infections, and in the brains of experimental animals infected from them.

4. Certain stages in the life-cycle of the protozoal parasite described appear to be 'filtrable'; it has been possible to produce evidence of infection in experimental animals by the inoculation of brain suspensions passed through Berkefeld V and N candles.

5. The protozoal parasite described appears to be a specific infection of the central nervous system, and, in spite of exhaustive searches, it has never been observed in other organs and tissues.

6. This parasite has never been observed except in association with natural or experimental rabies infection.

7. The possibility of the protozoal parasite described being connected with the ætiology of rabies has been discussed.

8. The possible relationship of this parasite to Negri bodies has also been discussed and the suggestion has been put forward that Negri bodies may represent a stage in the life-cycle of the parasite.

ACKNOWLEDGMENTS.

The author desires to express his thanks to Lieut.-Colonel K. R. K. Iyengar, O.B.E., I.M.S. (Retd.), Director, Pasteur Institute, Coonoor, for his valuable guidance and kind encouragement. His thanks are also due to his Laboratory Assistant, Mr. M. B. Ajjah, for his technical help during the conduct of this investigation.

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| Idem | (1924c) | .. | .. | <i>Ibid.</i> , 91 , p. 56. |

Addendum.—The following interesting observations were made after the paper was accepted for publication:—

1. Rajamanickam, aged 10 years, was bitten on the face by a rabid dog on 4th September, 1944. The wounds were cauterized within two hours after the bite and antirabic treatment was started immediately. The patient developed definite signs of hydrophobia on 25th September, 1944, and died two days later. This patient was infected at Kotagiri, Nilgiris District, S. India, where some of the strains of rabies virus reported in the paper

had originated. Saliva from this patient was inoculated into the neck muscles of guinea-pigs. One of them showed signs of rabies on the 52nd day and died the next day. Negri bodies and the parasite were found in the brain.

2. The different stages of the parasite described have since been encountered for the first time in the mid-brain of a cat naturally infected with rabies. The brain of a guinea-pig inoculated subdurally with a suspension of the brain of this cat also showed the parasite.

N. VEERARAGHAVAN.

21st November, 1914.

ISOLATION OF A STRAIN OF *RICKETTSIA ORIENTALIS* FROM CASES OF XK TYPHUS IN CEYLON.

BY

MAJOR R. B. LUCAS, R.A.M.C..

[Received for publication, July 16, 1944.]

It has been known for some years that diseases of the typhus group occur in Ceylon. As strains of *R. orientalis* have not previously been isolated and maintained in passage in laboratory animals, it is thought that the present account of the isolation of such a strain may be of some interest.

The first recorded instance of fevers of the typhus group in Ceylon is a description of two cases by Fernando (1938). These cases were of the X19 type and were apparently of murine origin. Wolff (1939) isolated a rickettsial strain from rat-fleas obtained from the house of one of these patients. Cases of the XK type were first described by Wijerama (1938). Blood from one case, when inoculated into a guinea-pig, gave rise to slight scrotal swelling and 'Rickettsia bodies were isolated from the peritoneal fluid'. Blood from the other case described was injected into the anterior chamber of a rabbit's eye and rickettsia bodies were demonstrated in scrapings of Descemet's membrane. The clinical findings in a further case of XK typhus are described by Nicholls (1940).

Despite the paucity of recorded cases in Ceylon, it is highly probable that numbers of cases of both these types of typhus occur, but are not diagnosed correctly. The majority of cases of 'fever' occurring out of Colombo are treated in rural hospitals or by private practitioners, and in such circumstances the application of laboratory procedures is not always possible. Since the arrival of military forces in number in Ceylon, sporadic cases of XK typhus have been observed from time to time among the troops, but the number was small till the beginning of this year when a considerable number of cases was admitted to the military hospitals. From these cases a strain of *R. orientalis* was isolated.

ANIMAL EXPERIMENTS.

The laboratory animals available at the time of occurrence of the cases were guinea-pigs and rabbits. White rats and white mice were not available till later, the mice not being obtained in time to inoculate them with blood from patients.

1. *Guinea-pigs*.—The results of infection of guinea-pigs with *R. orientalis* varies according to the virulence for these animals of the strain employed. In most cases the virulence is low.

In Malaya, Lewthwaite and Savoor (1936) injected a large number of guinea-pigs with blood from patients. Only after many such experiments were they able to isolate a strain which could be transmitted indefinitely in these animals. This strain then produced fatal results in 90 per cent of infected animals. Previous workers, they note, were unable to isolate strains, though febrile reactions were produced in guinea-pigs in some instances. Covell (1936) experienced similar difficulties in India, and was unable to isolate the virus from cases of XK typhus though some of his guinea-pigs reacted with fever. On the other hand, Kouwenaar and Wolff (1934) had found that the virus of Sumatran mite fever (the XK typhus of Sumatra) produced a severe illness in guinea-pigs with a mortality rate of 63 per cent. They note, however, that in Japan the virus produces only a mild reaction in these animals.

In the present experiments three series of guinea-pigs were used. In the first series 10 guinea-pigs were inoculated with blood taken from 12 cases of XK typhus, the day of disease varying between the 7th and the 14th. The blood was allowed to clot, the serum removed, and the clot then ground up in normal saline. The inoculum was 5 c.c. intraperitoneally. In two cases the inoculum consisted of pooled clot from two patients. The animals

rectal temperatures were taken twice daily. One guinea-pig only from this series was killed on the 8th day after inoculation and passage made with pooled brain and spleen suspension. The second animal was killed 21 days later and two subsequent passages at 12-day intervals were made. No febrile reactions were noted in any of these animals, there were no abnormalities found post mortem and no rickettsiae were found in scrapings from the peritoneum and tunica vaginalis. In those animals which were not killed the temperature record was maintained for 30 days after inoculation.

In the second series, two guinea-pigs were inoculated with pooled clot from two 12th day cases. Passages were made every 12th day for four generations. This series will be referred to later.

In the third series the animals were inoculated with material obtained at autopsy of fatal cases. One guinea-pig was inoculated with suspension of brain but died the following day from septic infection. Two guinea-pigs were inoculated with suspensions of lymph glands taken from a case fatal on the 18th day of the disease. These animals were killed 12 days after inoculation and passages made. One patient developed a splenic abscess which was aspirated twice, only blood and splenic tissue being obtained at the first aspiration. This material was inoculated into a guinea-pig and two subsequent passages made at 12-day intervals. No reactions were noted in any of the animals of this series.

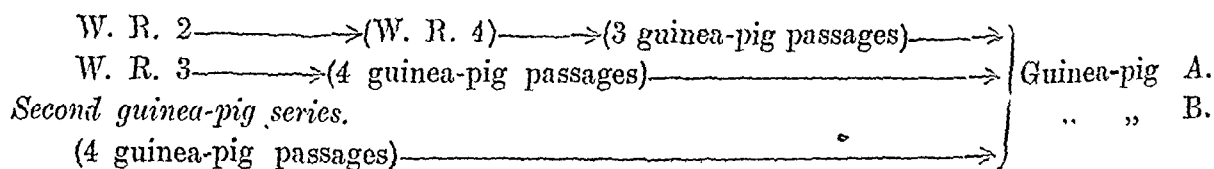
2. *Rabbits*.—Inoculation of infected material into the anterior chamber of the rabbit's eye causes an irido-cyclitis in from 4 to 15 days and rickettsiae can be demonstrated in the cells of the endothelium lining Descemet's membrane (Nagayo *et al.*, 1930). Lewthwaite and Savor report considerable success with this method, though they point out that there is difficulty in maintaining the strain through the first three generations. Wolff and Kouwenaar (1935), working with Sumatran cases, reported much less constantly positive results than were obtained by the Japanese workers and obtained only one positive reaction when using patients' blood.

In the work now described, four rabbits were inoculated in the anterior chamber with a suspension of ground-up blood clot in saline from four 12th day cases. One rabbit was inoculated with spleen suspension and one with brain suspension from a patient who had died on the 18th day. Four animals were inoculated with suspensions of lymph glands removed at biopsy from cases on the 24th day. No reactions were obtained in any of these animals.

3. *White rats*.—Lewthwaite and Savor (*loc. cit.*) showed that white rats could readily be infected with the virus they had already isolated, and that infection could be transmitted in series in these animals indefinitely. Infected rats showed little or no fever and there was no scrotal reaction. Rickettsiae could be demonstrated, though in very small numbers, in smears from the tunica vaginalis. Webster (1910), however, states that numerous rickettsiae may be found in scrapings from the tunica vaginalis of infected white rats.

In this work, three white rats were inoculated intraperitoneally with 4 c.c. crushed blood clot in saline from three cases on the 14th day of the disease. The first rat died within two hours of inoculation, the second died on the 19th day and the third was killed on the 21st day after inoculation. The second rat showed enlargement of the spleen at post-mortem examination, but no abnormalities were found in the third. Rickettsiae were detected in neither rat. Passages were made from these animals, and in addition, material from the second guinea-pig series was also incorporated. The position is shown in the following table. Such a pooling of material was dictated by the necessity, at this stage, for strict economy in guinea-pigs.

White rats.



All the guinea-pig passages shown in the table were made at 12- to 14-day intervals. None of the animals developed pyrexia or showed any other signs of infection. The last two guinea-pigs in the series, A and B, were killed 14 days after inoculation, the brains and spleens pooled, and 1 c.c. of suspension injected intraperitoneally into each of two white mice. The results of this will be discussed later.

4. *White mice*.—Dinger (1933) showed that white mice are very susceptible to infection with *R. orientalis*, and that practically all mice so infected die. The white mouse is thus the animal of choice for the isolation of strains of *R. orientalis* from cases of the disease or from other infected material. In the isolation of the virus from human cases 0.5 to 1 c.c. of crushed blood clot in saline, defibrinated blood or citrated blood from which the plasma has been removed is injected intraperitoneally. In passage experiments the usual suspensions of internal organs are used as the inoculum.

Pooled brain and spleen from guinea-pigs A and B were injected into two white mice. One of these died 17 days later and the other on the 18th day after inoculation. Autopsy showed in each case considerable enlargement of the spleen and an abundant, rather viscous peritoneal exudate. Smears from the peritoneum showed numerous *R. orientalis* within the cytoplasm of the endothelial cells. The strain has since been maintained in mice, death of the infected animal occurring in a period which has varied from 10 to 20 days, the average period being 15 days. A practical point in this respect is worth noting. On two occasions passage material was derived from mice which had died overnight and in which decomposition was just noticeable. The mice inoculated with material from these animals did not become infected with the rickettsial strain.

EXPERIMENTS WITH THE DEVELOPING CHICK EMBRYO.

Since rickettsiae of certain other diseases of the typhus group have been shown to grow well in the yolk sac of the developing chick embryo, an attempt was made to obtain a growth of *R. orientalis* in a similar manner.

Ten fertile hen's eggs, incubated for 6 days at 39°C., were inoculated according to Cox's (1941) technique with 0.5 c.c. blood clot in saline from 7th to 12th day cases. The eggs were then incubated at 37°C. Only three embryos died within 6 days of inoculation and in these cases bacterial infection was responsible. The remaining embryos continued to develop for some days, but eventually all died between the 9th and 14th days. They were found to be bacteriologically sterile and rickettsiae could not be demonstrated.

Gispen (1941) states that the virus of scrub typhus and Sumatran mite fever fail to grow on the chick-egg membrane, but grow well on the chorio-allantois of duck eggs. Possibly a similar state of affairs exists in regard to the yolk sac. It is also possible that the virus concentration in the blood of cases of the disease is insufficient to infect the yolk sac, whereas high concentrations such as are found in the tissues of passage animals might produce a successful result.

DISCUSSION.

The strain, finally isolated in white mice, was transmitted through either the rat series, the guinea-pig series, or through both. In any event the infection passed through at least four generations of guinea-pigs, these animals having shown no signs of infection whatever.

When guinea-pigs have failed to react with pyrexia after inoculation with infected material, it has usually been considered that such animals were of no further value in the isolation of the strain. Similarly, when passage animals have failed to show a pyrexial reaction after the initial animals in the passages had so responded, the experiments have usually been concluded.

The experiments just described indicate that though guinea-pigs may not react obviously to inoculation, yet the virus can ultimately be recovered after causing a transmissible inapparent infection, which has been shown to be capable of passing through at least four generations of guinea-pigs. Thus, where circumstances determine that guinea-pigs alone be

used in attempts to isolate strains of *R. orientalis*, passages should be maintained even though the animals show no obvious signs of infection.

In view of the success obtained with rabbits by numbers of workers, failure to infect these animals in the work just described requires an explanation. The cause may lie in the fact that the earliest case in which rabbits were used was a patient in the 12th day of the disease.

SUMMARY.

1. The isolation of a strain of *R. orientalis* is described.
2. The white mouse is considered to be the experimental animal of choice for this type of work.
3. Guinea-pigs, in the majority of cases, contract an inapparent infection when inoculated with *R. orientalis*. This state of inapparent infection has been transmitted through four generations of the animals.
4. Attempts to infect the yolk sac of the developing chick embryo failed.

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IN VITRO TEST FOR ASSAY OF POTENCY OF COBRA ANTIVENENE.

BY

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VENOMS of different species of snakes vary considerably in their toxicity, composition and antigenic structure. The toxic elements of some venoms are thermostable and are contained chiefly in the non-coagulable proteids (albumoses), e.g. venom of *Naia tripudians*; in others they are thermolabile and reside mainly in the coagulable proteids, e.g. venom of *Daboia russelli*. Some are rich in neurotoxins, others contain mainly cardio-vascular toxins.

Due to this complexity of the antigenic structure of venoms and the difficulty of obtaining standard preparations—venoms or antivenom sera—it has not been possible to lay down an international standard for the assay of potency of antivenom sera. Various institutes concerned in the preparation of antivenene adopt methods of assay which are considered to be most convenient and suitable for their particular products.

At the Central Research Institute, Kasauli, where a bivalent antivenom serum is prepared against cobra and daboia venoms, the routine assays are carried out by a biological method using pigeons of 290 to 320 grammes weight. For the estimation of the potency of cobra antivenom serum an arbitrary 'saving value' of not less than 0.5 mg. per c.c. of unconcentrated immune serum has been the standard adopted. This is calculated by deducting the minimum lethal dose of cobra venom for a pigeon, from the maximum amount of venom that a pigeon can withstand, when the mixture of venom plus 1 c.c. of antiserum is injected intramuscularly into the pectoral muscles of the bird. For example, if the m.l.d. of venom is 0.3 mg. and the pigeon receiving 0.8 mg. plus 1 c.c. of antiserum survives, the saving value of 1 c.c. of serum is taken as 0.5 mg. of cobra venom. This *in vivo* test is not entirely satisfactory. Long experience has shown that the susceptibility of individual pigeons to cobra venom varies considerably. For example, the same sample of venom may kill some pigeons of standard weight in a dose of 0.3 mg., others in a dose of 0.4 mg., while in some cases pigeons receiving 0.4 mg. may survive and those receiving 0.3 mg. die. Similar results are at times obtained when venom plus antiserum is injected for routine testing, pigeons receiving 0.9 mg. of cobra venom plus 1 c.c. of antiserum may live, while pigeons receiving 0.8 mg. plus 1 c.c. of antiserum may die, the serum and the venom in both cases being the same. In order to obtain accurate readings it would, therefore, be necessary to employ a large number of birds for each individual test. This is not always practicable because of the difficulty of obtaining a sufficient number of pigeons of standard weight.

It is obvious, therefore, that a very real need exists for a more reliable method for the routine assay of antivenom sera.

In testing the hæmolytic effects of cobra and daboia venoms in presence of normal horse serum and sera of horses immunized against cobra and daboia venoms, it was observed that the washed blood cells of certain species of animals, normally hæmolyzed by those venoms, were not hæmolyzed in the presence of immune sera. This observation suggested the possibility of utilizing the inhibitory effect of antivenom serum on hæmolysis as a measure of its potency in neutralizing the effect of the corresponding venoms. A similar test was used by Lamb (1904) to test the specificity of cobra antiserum against the hæmolytic action of

the venoms of other snakes, and a modification of his technique was employed in the test to be described.

Preliminary experiments were carried out to determine the hæmolytic activity of cobra venom for guinea-pig cells, details of which are given in Table I. Six different batches of cobra venom were tested. Each of these batches was composed of a mixture of the venom extracted from several cobras and may therefore be regarded as average samples. The results recorded in Table I show that very minute doses of cobra venom are capable of hæmolyzing guinea-pig cells :—

TABLE I.
Hæmolytic effect of cobra venom on guinea-pig cells.

Cobra venom (mg.)	0.1	0.08	0.06	0.04	0.02	0.01	0.005
Normal saline solution (c.c.)	1	1	1	1	1	1	1
3 per cent suspension guinea-pig cells (c.c.)	1	1	1	1	1	1	1
Batch A	++	++	++	++	—	—
„ B	++	++	++	++	—	—
„ C	++	++	++	++	++	—
„ D	++	++	++	++	—	—
„ E	++	++	++	++	—	—
„ F	++	++	++	++	+	—

++ = complete hæmolysis ; + = partial hæmolysis ; — = no hæmolysis.

In the *in vitro* test to be described guinea-pig cells were used as the indicator of hæmolysis. The technique of the test is as follows :—

To a series of tubes (convenient size $2\frac{1}{2}'' \times \frac{1}{2}''$) cobra venom and the serum under test are added in varying proportions as shown in Table II. The mixture is shaken and incubated at 37°C. for 1 hour, at the end of which period 1 c.c. of 3 per cent suspension of washed guinea-pig erythrocytes is added to each tube. The mixture is shaken to ensure that the fluids in the tubes are thoroughly mixed and, after standing for 18 hours at room temperature, the readings are recorded.

TABLE II.
Standard protocol for hæmolytic test.

	Tube 1.	Tube 2.	Tube 3.	Tube 4.	Tube 5.	Tube 6.	Tube 7.	Tube 8.
Cobra venom 1 mg. } per c.c. in normal } c.c. saline.	0.1	0.2	0.3	0.4	0.5	0.6	0.75	1
Normal saline solu- } tion. } c.c.	0.9	0.8	0.7	0.6	0.5	0.4	0.25	..
Serum under test	c.c. 1	1	1	1	1	1	1	1
Shake and incubate for 1 hour at 37°C.								
3 per cent guinea- } pig cells in normal } c.c. saline.	1	1	1	1	1	1	1	1

Eight normal horses were given a series of gradually increasing doses of cobra venom and their sera were tested at weekly intervals for presence of anti-hæmolytic activity as estimated by the *in vitro* test. Six to eight weeks after the commencement of immunization all the horses showed a measurable response. 1 c.c. of serum inhibiting the hæmolytic effect of 0.1 mg. to 0.2 mg. of cobra venom. With the increase in immunizing dose of cobra venom there was a further rise in the anti-hæmolytic titre, sera of some horses neutralizing as much as 0.5 mg. of cobra venom at the end of 3 months.

Forty-four samples of sera from 19 horses at various stages of immunization against cobra venom were tested in parallel by the *in vitro* test here described and by the *in vivo* test in routine use at Kasauli. The sample of venom used in the *in vitro* test had a minimum hæmolytic dose of 0.03 mg. for 1 c.c. of 3 per cent guinea-pig cells, and that used for the *in vivo* test had a minimum lethal dose of from 0.3 mg. to 0.4 mg. for pigeons of standard weight. The anti-hæmolytic potency of the various sera tested varied and according to the results obtained they have been arranged in three different categories in Table III (groups A, B and C). In group A hæmolysis—partial or complete—occurs with 0.4 mg. or less of venom. In group C partial or no hæmolysis occurs with 0.6 mg. of venom, and in the intermediate group B partial or complete hæmolysis occurs with 0.5 mg.

TABLE III.
Comparative results of in vitro and in vivo tests.

Date of test.	Horse number.	IN VITRO TEST.								IN VIVO TEST.		
		COBRA VENOM (MG.).								COBRA VENOM (MG.).		
		0.1	0.2	0.3	0.4	0.5	0.6	0.75	1	0.7	0.8	0.9
<i>Group A.</i>												
10-7-44	37	—	+	++	++	+++	++	++	++	D	D	D
19-7-44	37	—	+	++	++	+++	++	++	++	D	D	D
16-6-44	5	—	—	++	++	++	++	++	++	L	D	D
19-7-44	5	—	—	++	++	++	++	++	++	L	D	D
23-6-44	25	—	—	+	++	++	++	++	++	D	D	D
23-6-44	25	—	—	+	++	++	++	++	++	D	D	D
16-6-44	26	—	—	+	++	++	++	++	++	L	L	D
26-7-44	26	—	—	+	++	++	++	++	++	D	D	L
10-7-44	21	—	—	+	++	++	++	++	++	D	D	D
26-7-44	21	—	—	+	++	++	++	++	++	D	D	D
19-7-44	36	—	—	+	++	++	++	++	++	D	D	D
26-7-44	35	—	—	+	+	++	++	++	++	D	D	D
10-7-44	36	—	—	—	+	++	++	++	++	D	D	D
26-7-44	36	—	—	—	+	++	++	++	++	D	D	D
22-5-44	25	—	—	—	++	++	++	++	++	D	D	D
13-5-44	31	—	—	—	++	++	++	++	++	D	D	D
13-5-44	26	—	—	—	++	++	++	++	++	L	L	L
13-5-44	5	—	—	—	++	++	++	++	++	L	L	L
6-6-44	21	—	—	—	++	++	++	++	++	D	L	D

++ = complete hæmolysis.
+ = partial hæmolysis.
— = no hæmolysis.

L = lived.
D = died.

TABLE III—*concl'd.*

Date of test.	Horse number.	IN VITRO TEST.								IN VIVO TEST.		
		Cobra Venom (mg.).								Cobra Venom (mg.).		
		0.1	0.2	0.3	0.4	0.5	0.6	0.75	1	0.7	0.8	0.9
<i>Group B.</i>												
16-6-44	2	—	—	—	—	++	++	+++	++	L	L	L
13-5-44	2	—	—	—	—	..	++	+++	+++	..	L	L
16-6-44	4	—	—	—	—	+	+	+++	+++	L	L	D
16-6-44	17	—	—	—	—	+	++	+++	++	L	L	D
6-6-44	30	—	—	—	—	++	++	++	++	..	L	L
6-6-44	11	—	—	—	—	++	+++	+++	++	..	L	L
13-5-44	25	—	—	—	—	++	++	+++	+++	..	D	D
<i>Group C.</i>												
26-7-44	31	—	—	—	—	—	+	+++	++	..	L	L
16-6-44	6	—	—	—	—	—	+	++	++	..	L	D
23-6-44	15	—	—	—	—	—	+	++	++	..	L	L
22-5-44	15	—	—	—	—	—	+	++	++	..	L	L
22-5-44	31	—	—	—	—	—	—	+	++	..	L	L
16-6-44	27	—	—	—	—	—	—	+	++	..	L	L
13-5-44	17	—	—	—	—	—	—	+	++	..	L	D
13-5-44	1	—	—	—	—	—	—	—	++	..	L	L
6-6-44	22	—	—	—	—	—	—	—	++	..	L	L
13-5-44	27	—	—	—	—	—	—	—	++	..	L	L
10-7-44	22	—	—	—	—	—	—	—	+	..	L	L
26-7-44	27	—	—	—	—	—	—	—	+	..	L	L
26-7-44	20	—	—	—	—	—	—	—	—	..	L	L
23-6-44	20	—	—	—	—	—	—	—	—	..	L	L
22-5-44	20	—	—	—	—	—	—	—	—	..	L	L
30-5-44	13	—	—	—	—	—	—	—	—	..	L	L
13-5-44	6	—	—	—	—	—	—	—	—	..	L	L
26-7-44	15	—	—	—	—	—	—	—	—	..	L	L

++ = complete hæmolysis.
 + = partial hæmolysis.
 — = no hæmolysis.

L = lived.
 D = died.
 .. = not tested.

DISCUSSION.

The results show that there is a fairly well-marked correlation between the *in vitro* and *in vivo* tests. In the case of sera where hæmolysis is prevented up to a maximum of 0.3 mg. of cobra venom, as in group A (Table III), the serum fails to pass the *in vivo* test. Where 0.4 mg. to 0.5 mg. of cobra venom are neutralized per 1 c.c. of serum, as in group B, the serum may or may not pass the *in vivo* test, and when over 0.5 mg. of cobra venom is neutralized per c.c. of serum, as in group C, the serum passes the *in vivo* test. On the basis of these findings it can be stated that, as a rule, if the anti-hæmolytic titre of a serum is less than 0.4 mg. per c.c., it is not up to standard titre ; if it is over 0.5 mg. per c.c. it is up to titre ; and if it is between 0.4 mg. and 0.5 mg. per c.c. it is on the border line and may or may not be up to titre. It should, however, be noted that, while there appears to be a close parallelism between the two tests, occasional anomalous results are obtained, e.g. sera of horses Nos. 5 and 26, included in group A (Table III), pass the *in vivo* test but fail to pass the *in vitro* test.

It is known, as has been pointed out above, that the *in vivo* test is liable to a considerable margin of error on account of the variable susceptibility of individual pigeons. The method of estimating the reliability of the *in vitro* test described above was a comparison with the *in vivo* test in routine use. Although the result of the two tests carried out in parallel corresponded closely, such discrepancies as occurred may have been due to the known variability of the *in vivo* test. It is, indeed, possible that the *in vitro* test may be more constant and reliable than the *in vivo* test, but this question will require further work. For routine titrations of sera of horses under immunization against cobra venom the *in vitro* test has now been adopted. The final assay of potency of antivenene before issue for therapeutic use is, however, still carried out by the *in vivo* test. Further work may show the *in vitro* test to be equally or even more satisfactory for this purpose. The saving in pigeons effected by the use of the *in vitro* test for routine intermediate titrations makes it possible to employ a larger number of pigeons for final titration.

Experiments on similar lines are in progress for the assay of potency of daboia antivenom by *in vitro* methods.

CONCLUSIONS.

An *in vitro* test is described for the estimation of the anti-hæmolytic titre of cobra antivenene. The anti-hæmolytic potency of the immune serum corresponds closely with its anti-neurotoxic titre as determined by the *in vivo* test on pigeons.

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STUDIES ON EXPERIMENTAL FLUORINE POISONING IN RATS.

BY

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IN a previous communication (Ranganathan, 1941), it was recorded that calcium, administered either as a salt or as present in combination in natural foodstuffs, exerted a mitigating influence on fluorine poisoning in rats, and that, at the levels of dosage used, the toxic effect of fluorine was inversely proportional to calcium intake. In the present paper some further experiments on the effect of calcium salts on fluorine poisoning are described, together with other experiments which have a bearing on the problem of fluorosis.

EXPERIMENTAL.

The influence of different calcium salts.—It was previously shown that the administration of calcium lactate prolonged the survival period of rats given a lethal dose of fluorine. The action of a few other salts of calcium has now been investigated. Young rats of about 50 g. in weight were put on diets containing excess of fluorine to which various calcium salts were added, the doses of the latter being calculated on the basis of a similar intake of calcium. The criterion of the protection conferred by the salt was the survival period of the animals, and, to a lesser extent, their growth response.

Seven groups of rats, each including 8 animals, were fed on the following diets :—

GROUP I.—A basal diet (starch 60 parts, casein 20, gingelly oil 8, cod-liver oil 2 and dried yeast 5) *plus* 0.05 per cent sodium fluoride *plus* 5 per cent of a salt mixture containing iron citrate 3.54 parts, potassium phosphate 28.62, sodium phosphate 10.41, magnesium sulphate 7.98, sodium chloride 5.19 and potassium iodide 0.5. This salt mixture was calcium-free. The calcium content of this diet was 0.05 per cent.

GROUP II.—Basal diet *plus* 0.05 per cent sodium fluoride *plus* 5 per cent of a salt mixture containing calcium lactate 50.4 parts and other salts in the same proportions as in group I.

GROUP III.—Basal diet *plus* 0.05 per cent sodium fluoride *plus* 5 per cent of a salt mixture containing 97.0 parts of calcium gluconate and other salts in the same proportions as in group I.

GROUP IV.—Basal diet *plus* 0.05 per cent sodium fluoride *plus* 5 per cent of a salt mixture containing 23.9 parts of calcium phosphate and other salts in the same proportions as in group I.

GROUP V.—Basal diet *plus* 0.05 per cent sodium fluoride *plus* 5 per cent of a salt mixture containing 17.1 parts of calcium oxide and other salts in the same proportions as in group I.

GROUP VI.—Basal diet *plus* 0.05 per cent sodium fluoride *plus* 5 per cent of a salt mixture containing 23.1 parts of calcium carbonate and other salts in the same proportions as in group I.

GROUP VII.—Basal diet *plus* 0.05 per cent sodium fluoride *plus* 5 per cent of a salt mixture containing 25.7 parts of calcium chloride and other salts in the same proportions as in group I.

The calcium content of the diets of groups II to VII was the same, being 0.47 per cent. The experiments were continued for 82 days, when the surviving animals were killed. The

average survival period of the experimental animals in the various groups and the average weekly change in body-weight during the period of survival are shown in Table I:—

TABLE I.

Survival of rats as influenced by sodium fluoride and various salts of calcium.

Group.	NUMBER OF ANIMALS SURVIVED AFTER					Average survival period, days.	Average weekly change in body-weight, in g., during the period of survival.
	15	30	45	60	82 days.		
I ..	4	0	0	0	0	15	—2.0
II ..	7	7	7	7	7	73	10.5
III ..	7	7	7	7	7	73	5.3
IV ..	8	5	5	0	0	56	1.0
V ..	8	8	8	8	8	82	5.7
VI ..	8	8	8	7	7	79	6.2
VII ..	8	8	8	4	3	63	4.3

It will be seen from Table I that the various calcium salts, when given in quantities calculated to maintain a constant level of calcium in the diets containing lethal doses of fluorine, have roughly the same potency in mitigating the toxic effects of fluorine.

The effect of salts other than those of calcium.—The effect of magnesium, barium and aluminium salts, salts of elements which form insoluble fluorides, was then tried. Neither aluminium nor barium salts afforded any protection; all the experimental animals died within 7 days, roughly within the same period in which the negative controls died. These salts were probably themselves toxic. The magnesium salt, however, afforded some protection: the experimental animals receiving a supplement of magnesium carbonate survived on an average for 31 days as against an average survival of 43 days in the case of animals receiving a supplement of calcium lactate: this experiment was continued for 62 days when the surviving animals were killed, none in the magnesium group and 4 in the calcium group.

Relative toxicities of the fluorides of sodium, calcium and magnesium.—The following experiments were conducted to find out the relative toxicities of the three fluorides, the fluorides of sodium, calcium and magnesium, and also to ascertain if their relative toxicities could be correlated with their solubility in water. Young, growing albino rats of about 50 g. body-weight were used in groups of 6 animals each. Five groups were used and the experiments were continued for 100 days when the surviving animals were killed. The diet of the various groups and the survival period of the animals are given in Table II:—

TABLE II.

Relative toxicities of NaF, CaF₂ and MgF₂.

Group.	Diet.	NUMBER OF ANIMALS SURVIVED AFTER			Average survival period, days.
		10	50	100 days.	
I ..	Basal diet plus calcium-free salt mixture plus 0.05 per cent sodium fluoride.	0	0	0	7
II ..	Same as group I but with calcium fluoride containing the same amount of fluorine as in group I.	6	4	4	74

TABLE II—*concd.*

Group.	Diet.	NUMBER OF ANIMALS SURVIVED AFTER			Average survival period, days.
		10	50	100 days.	
III	.. Same as group I but with calcium fluoride containing 4 times the amount of fluorine as in group I.	2	2	2	37
IV	.. Same as group I but with magnesium fluoride containing the same amount of fluorine as in group I.	0	0	0	5
V	.. Same as group I but with magnesium fluoride containing 4 times the amount of fluorine as in group I.	0	0	0	5

It is to be seen from Table II that magnesium fluoride appeared to be the most toxic followed in descending order by sodium fluoride and calcium fluoride. Of the three fluorides investigated, sodium fluoride is soluble, while the fluorides of calcium and magnesium are insoluble in water. Yet magnesium fluoride, an insoluble fluoride, was the most toxic. It is, therefore, obvious that the relative toxicity of fluorine compounds cannot be accounted for by their solubility in water.

Further experiments were carried out to ascertain whether the addition of a calcium or a magnesium salt to animals receiving calcium fluoride or magnesium fluoride along with a basal diet and calcium-free salt mixture had any beneficial effect in lessening the severity of fluorine poisoning. It was found that the addition of calcium lactate to animals receiving either calcium fluoride or magnesium fluoride helped in mitigating the toxic effect of fluorine, while the addition of a magnesium salt to animals receiving magnesium fluoride was not so helpful. These results again bring out the beneficial rôle of calcium in fluorine poisoning.

Vitamin C and fluorine poisoning.—Pandit, Raghavachari, Subba Rao and Krishnamurthy (1940) claimed that deficiency of vitamin C is a contributory factor in the production of chronic fluorine intoxication in man. They based this conclusion on the computation of the vitamin C intake of human beings in an area in which fluorosis is endemic, calculated intake being compared with a very high standard of normal requirements (170 mg. daily for an adult). Pandit and Narayana Rao (1940) further recorded that a high intake of vitamin C, given in the form of fresh vegetables, mitigated the effects of fluorine poisoning in monkeys.

Experiments were carried out here in which additional vitamin C was given to rats receiving a toxic dose of fluorine. The rat is a species not normally requiring a supply of vitamin C through the diet. The addition to the basal diet containing sodium fluoride and the calcium-free salt mixture of large amounts of vitamin C (10 mg. per rat daily) had no effect on the survival rate, the animals dying as rapidly as negative controls not receiving a supplement of vitamin C. In the same series of experiments, the protective effect of a calcium salt was again observed. It should be mentioned that in the experiments of Pandit and Narayana Rao, the criterion of fluorine poisoning was radiological changes in the bones, while in the rat experiments here the criterion was the survival rate of the animals.

Vitamin D and fluorine poisoning.—Fluorine is stated by Morgareidge and Finn (1940) to hinder the process of healing in rachitic rats treated with cod-liver oil and with pure vitamin D. According to these authors, when a small dose of vitamin D and graded doses of fluorine were simultaneously given to groups of rats previously rendered rachitic, the healing process was hindered by fluorine, the effect being greater with increased dosage. Experiments conducted to verify the above observations clearly demonstrated the inhibitory effect of fluorine in the cure of rachitic conditions in rats by vitamin D. Animals receiving fluorine and adequate amounts of calcium in the diet, with or without vitamin D, had extensive hæmorrhages in the calcifying areas of the bones, and hypertrophy of the epiphyseal ends.

Calcification was uniformly poor, indicating that vitamin D had no beneficial effect on rachitic conditions superimposed on fluorosis, and confirming the findings of Morgareidge and Finn (*loc. cit.*).

DISCUSSION.

The present experiments have reinforced the previous finding that calcium salts have a mitigating effect on fluorine poisoning in rats. Magnesium salts confer a similar protection, though to a somewhat smaller degree. It is, however, not yet clear how the calcium or magnesium salts act in lessening the severity of fluorine poisoning. The beneficial effect observed with these salts cannot be wholly explained as being due to the mechanical removal of the fluorine as an insoluble fluoride. Attempts were made to throw light on this problem by a series of experiments in which a toxic dose of sodium fluoride administered orally was followed by either oral or subcutaneous administration of calcium lactate or calcium gluconate. Though these experiments were not very successful, they helped to show that calcium salt administered subcutaneously was equally efficacious in mitigating the toxic effects of fluorine. It is, therefore, obvious that there is something more than mere mechanical removal of the fluorine as an insoluble fluoride that is responsible for lessening the severity of fluorine poisoning. How exactly the protective effect of calcium is exerted remains to be explained.

SUMMARY AND CONCLUSIONS.

1. Further experiments were carried out on the effect of calcium salts on fluorine poisoning.
2. The different salts of calcium have roughly the same potency in mitigating the toxic effects of fluorine. Magnesium salts too confer a similar protection, though to a somewhat smaller degree.
3. The toxicity of fluorine compounds cannot be accounted for by their solubility in water; magnesium fluoride was found to be the most toxic, followed in descending order by sodium fluoride and calcium fluoride.
4. Vitamin C did not lessen the severity of fluorine poisoning in rats.
5. Vitamin D had no beneficial effect on rachitic conditions superimposed on fluorosis.

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STUDIES IN VITAL CAPACITY.

BY

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It has been pointed out by Dreyer (1919) that variations in vital capacity may be due to a racial factor. This view was supported by Wilson and Edwards (1921). During the last two decades, the question of racial factor, and the value of vital capacity as a test for physical fitness and efficiency, attracted the attention of the medical scientists in China, Japan, the Philippines and India. McCloy (1927) studied the problem in 500 Chinese students and found that 'there was a larger variation in nationals of one kind caused by climate, activity and body-build than is found between nations and races'. Krishnan and Vareed (1932, 1933) from their observations on 301 medical students at Madras expressed the opinion that 'the lower values obtained among South Indians might be due to warm climate, which is responsible for less tendency for exercise, low metabolism and poor chest expansion, but not due to the influence of race or nationality'. On the other hand, Bhatia (1929) working in Bombay supported the view of Dreyer. Mason (1932) from her extensive studies on South Indian women observed that both physical activity, especially during childhood, as well as race, might influence vital capacity. The racial factor in vital capacity is still a doubtful one.

Whatever the causative factor may be, it has been stressed by different investigators that variations are sufficiently wide to demand different standards for different countries. In India, Bhatia (*loc. cit.*) obtained results from a study on 100 healthy male subjects in Bombay. He found that 'standards according to body-surface area and standing height are most constant'. Krishnan and Vareed (*loc. cit.*) collected data on 301 subjects and found a definite correlation between vital capacity and standing height and chest expansion. Reddy (1933) recorded his observations on the vital capacity of 105 male students at Vizagapatam. De and De (1939) noted the vital capacity of 100 male subjects in Bengal.

A statistical study of the problems was first undertaken by Mason (*loc. cit.*). The observations were made on 587 women from different parts of South India. She correlated vital capacity with height, weight and surface area and devised standards for predicting vital capacity from these measurements. Similarly, Telang and Bhagwat (1941), working in Bombay on 172 male subjects, found a correlation between vital capacity and standing height, sitting height, weight, body-surface area, trunk-surface area, deflated chest, inflated chest, mean chest and chest expansion. They found no correlation with age. They stated that the most significant correlation was, firstly, with trunk surface and next with sitting height.

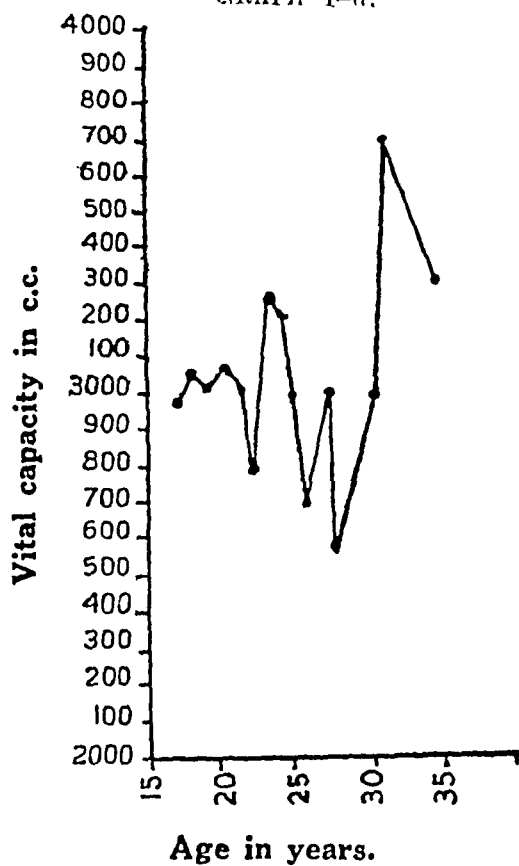
The data recorded in this paper are presented to show the range of vital capacity in healthy individuals in the Vizagapatam area and the relation of vital capacity to different anatomical measurements as obtained from these data.

Data obtained by other workers in India and the Indian total average and other non-Indian averages have been tabulated (Table II).

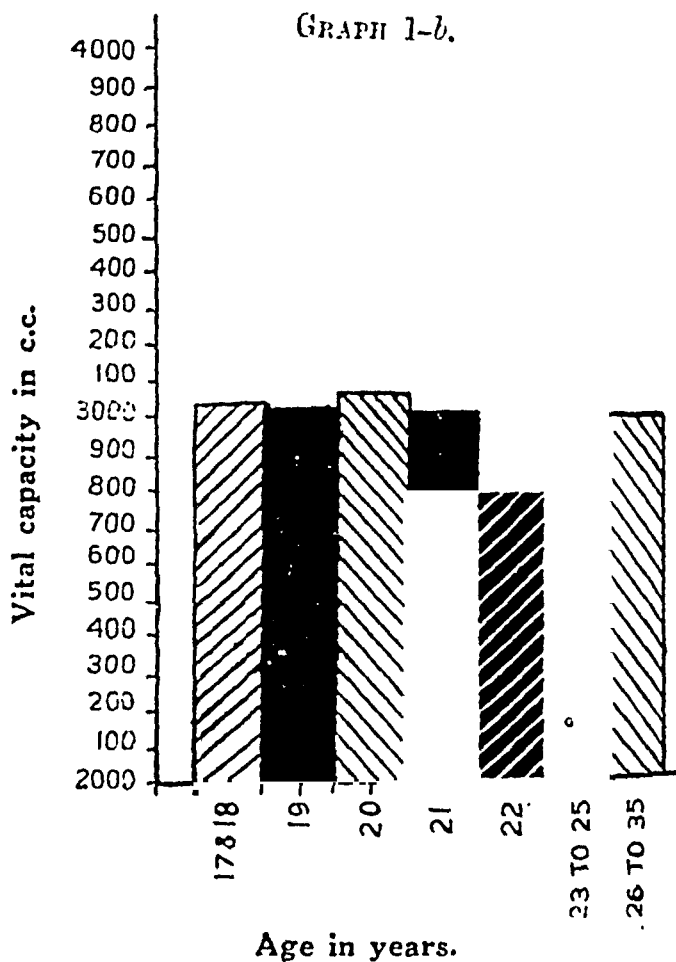
MATERIAL OF THE PRESENT INVESTIGATION.

The total number of individuals in whom vital capacity was measured was 310. The ages of these ranged between 17 and 43 years. Of these, 243 were students between the ages

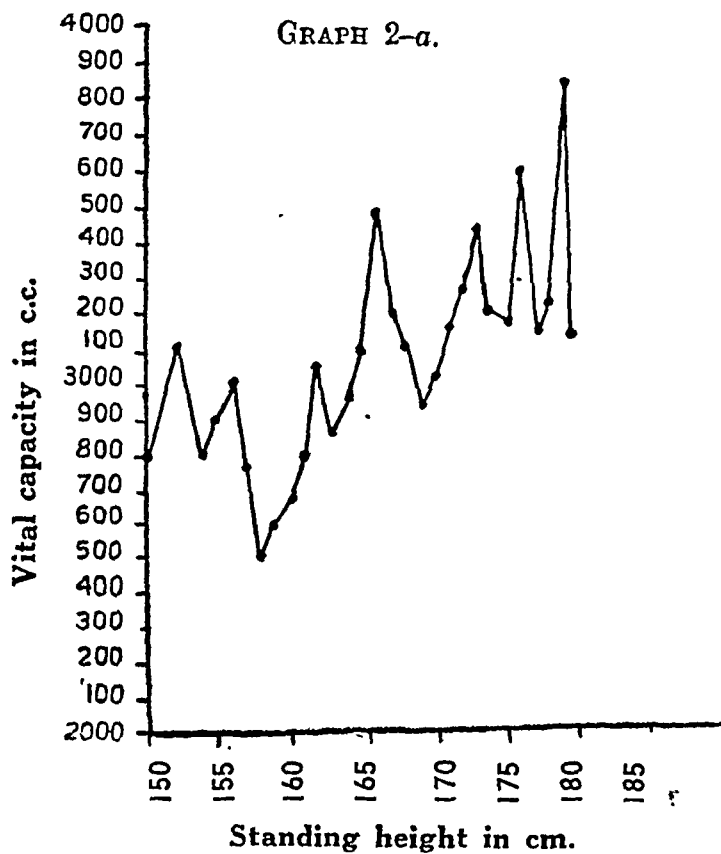
GRAPH 1-a.



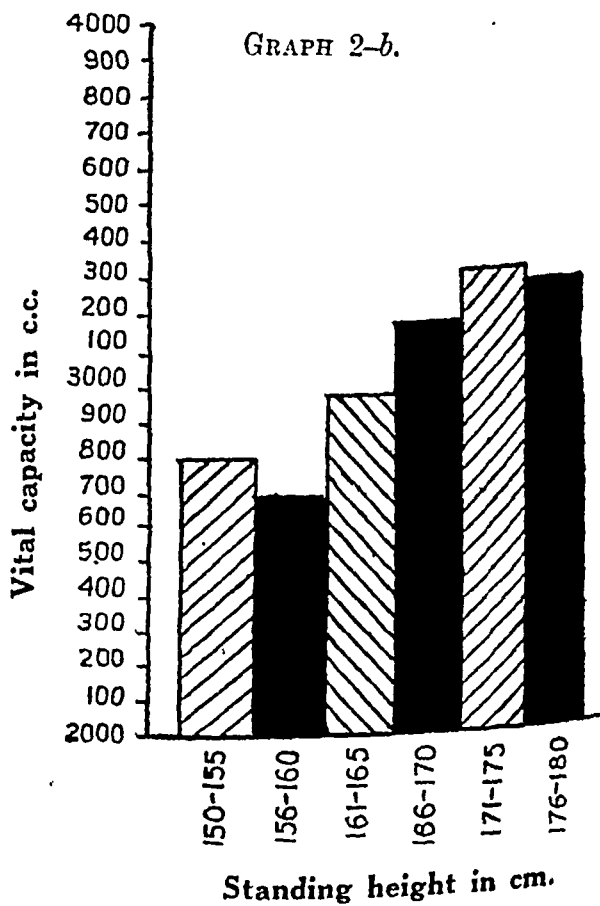
GRAPH 1-b.

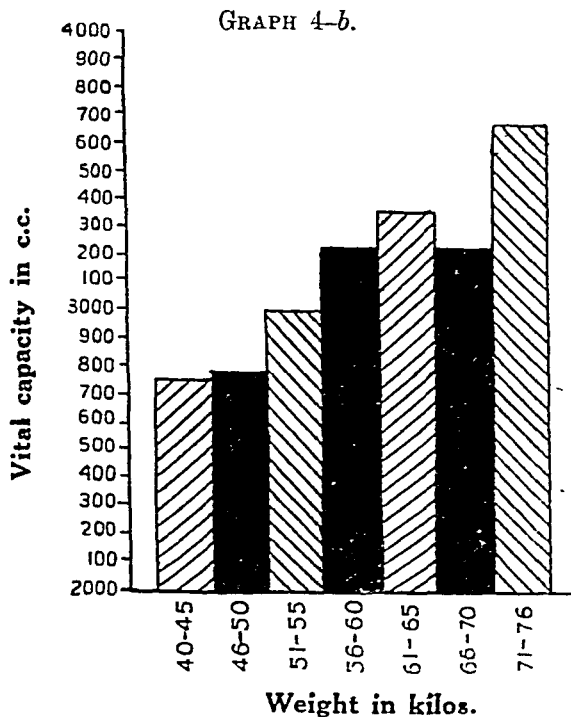
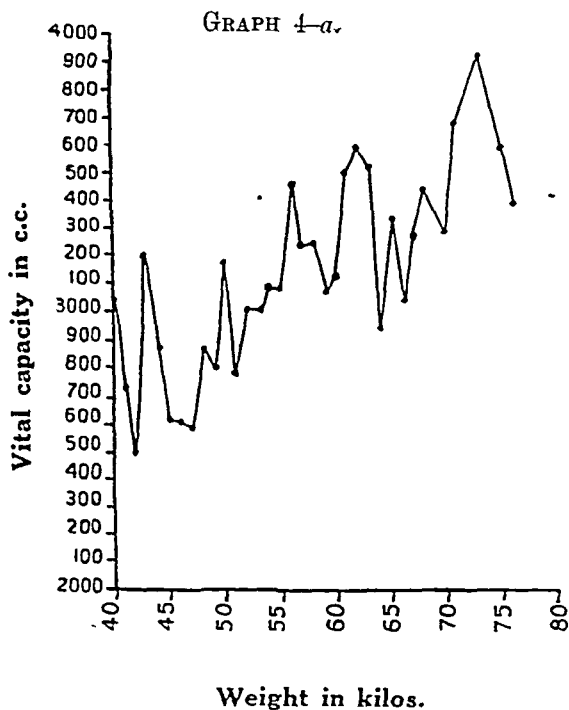
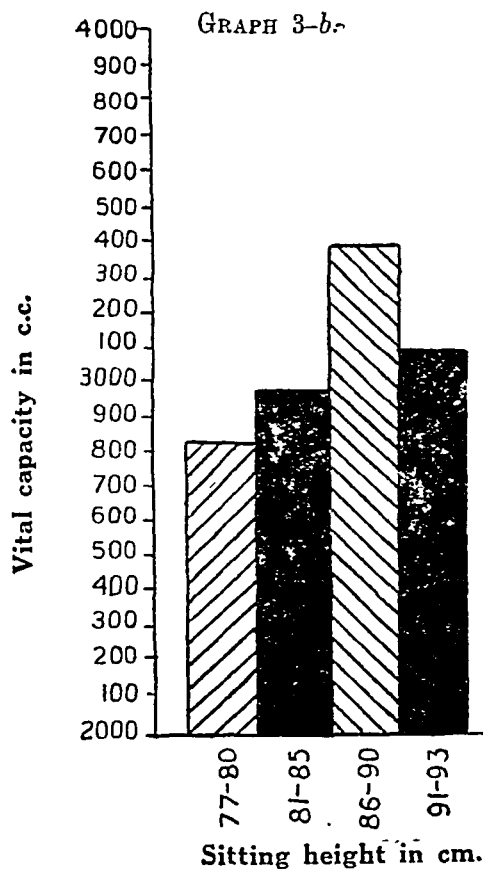
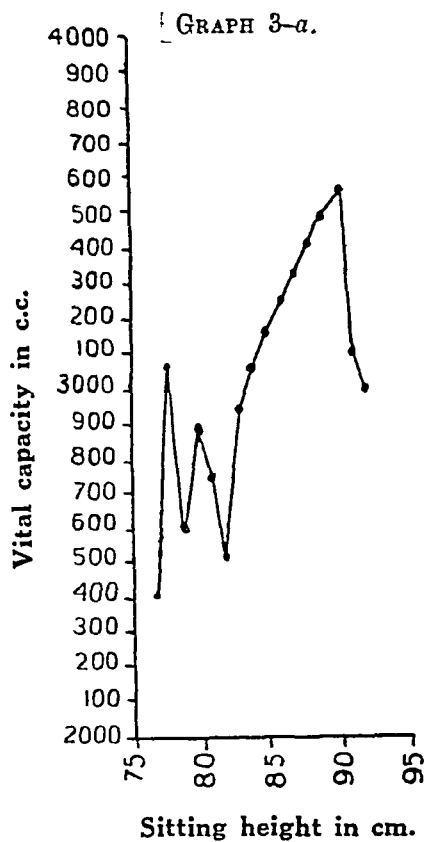


GRAPH 2-a.

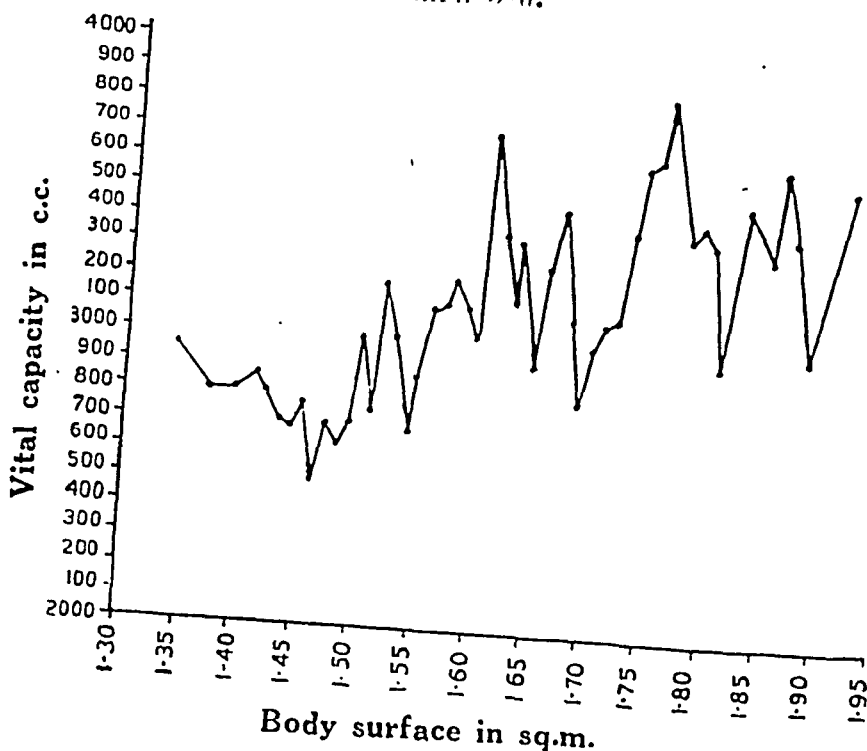


GRAPH 2-b.

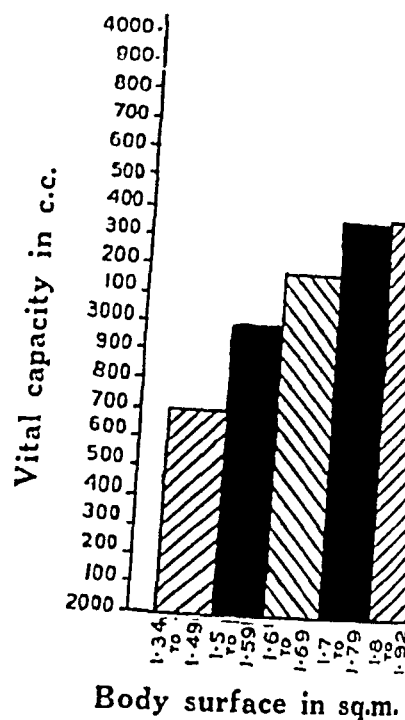




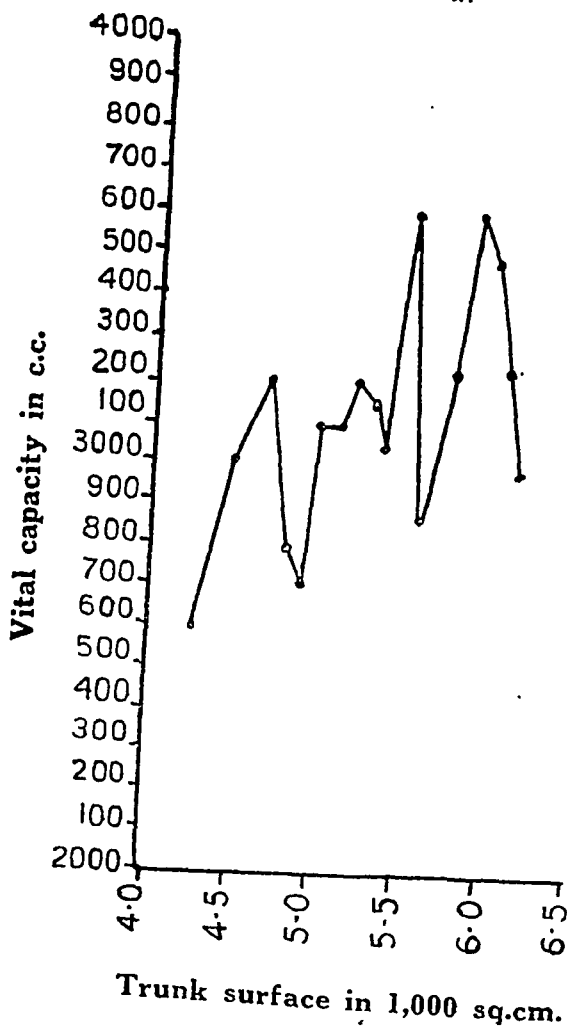
GRAPH 5-a.



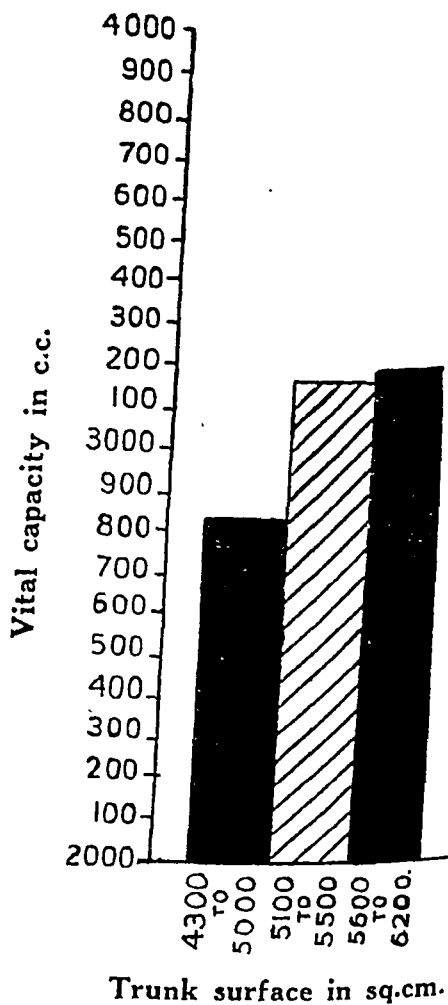
GRAPH 5-b.



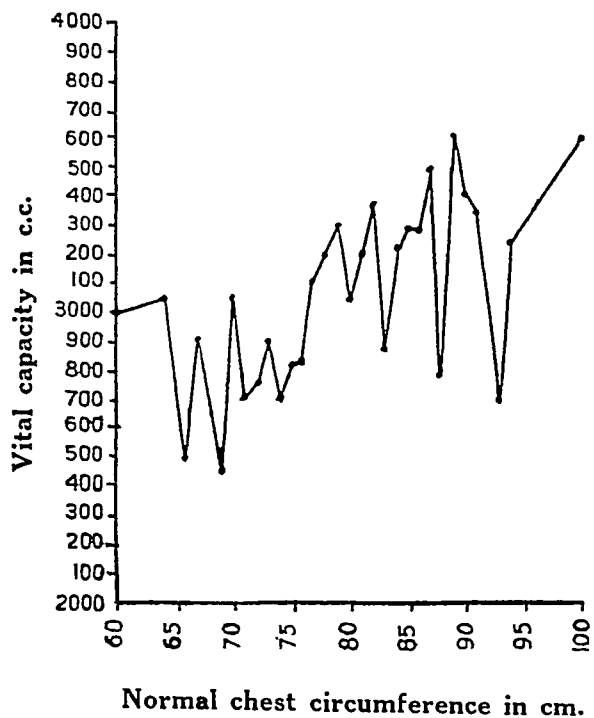
GRAPH 6-a.



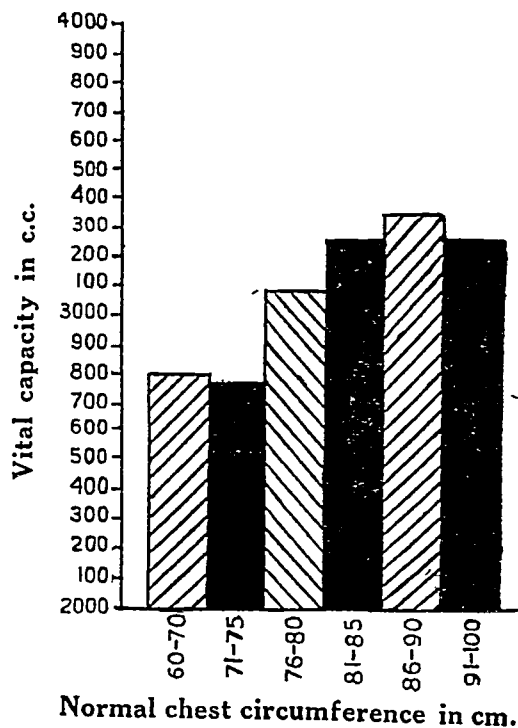
GRAPH 6-b.



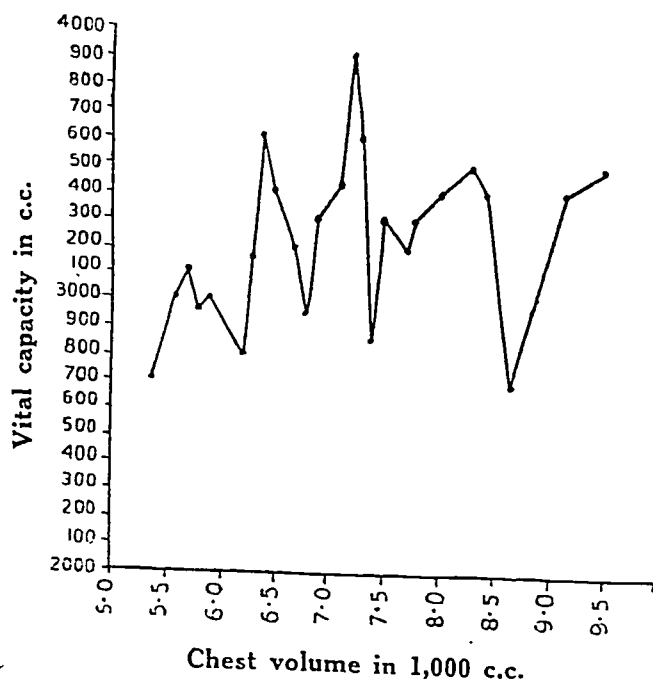
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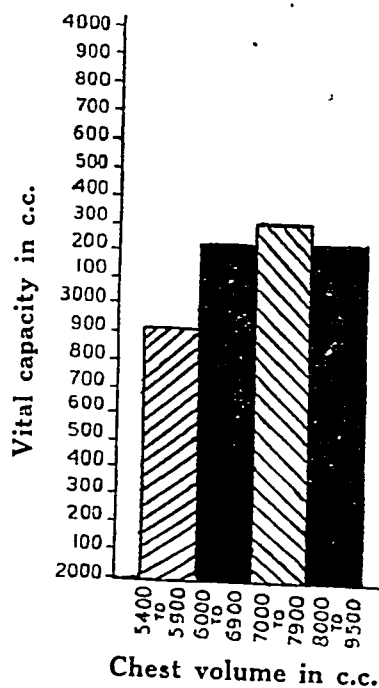
GRAPH 7-b.



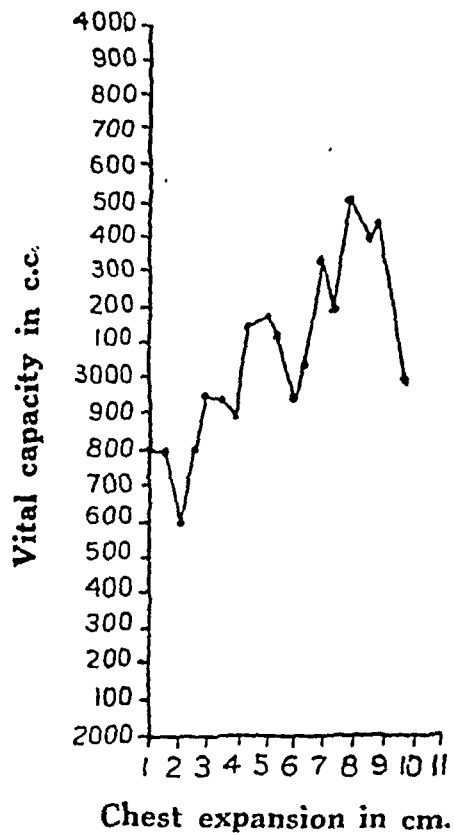
GRAPH 8-a.



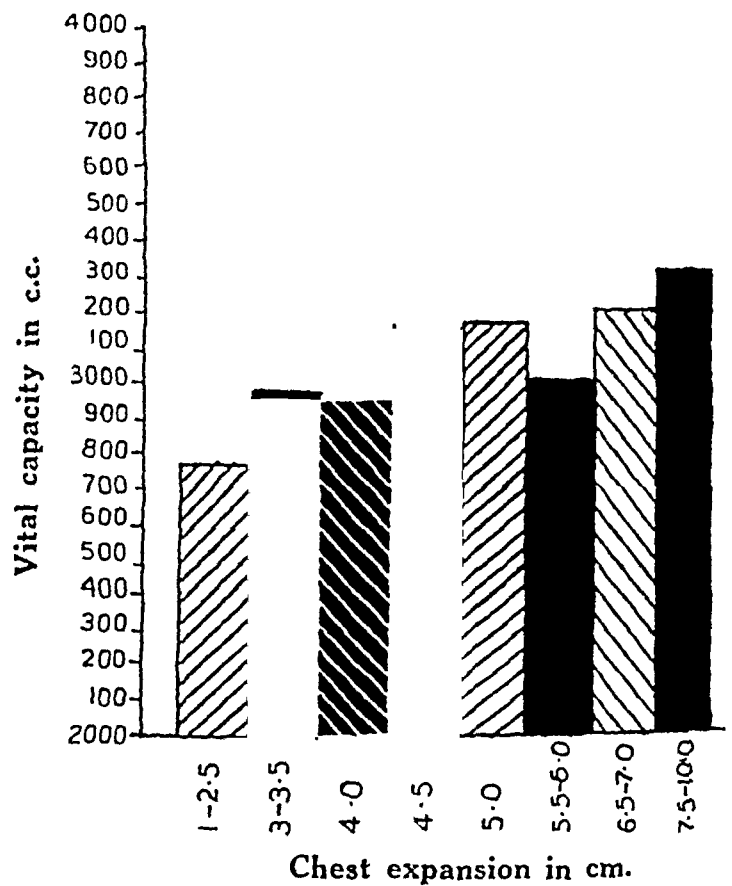
GRAPH 8-b.



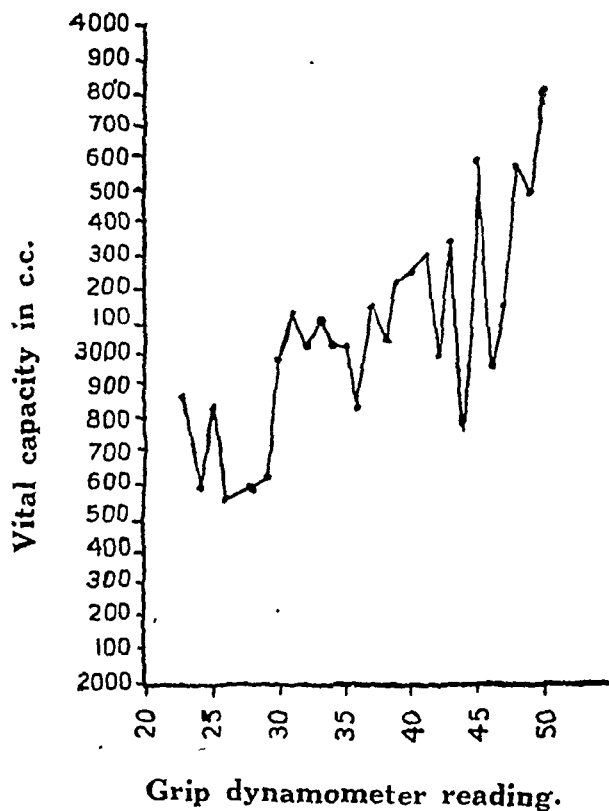
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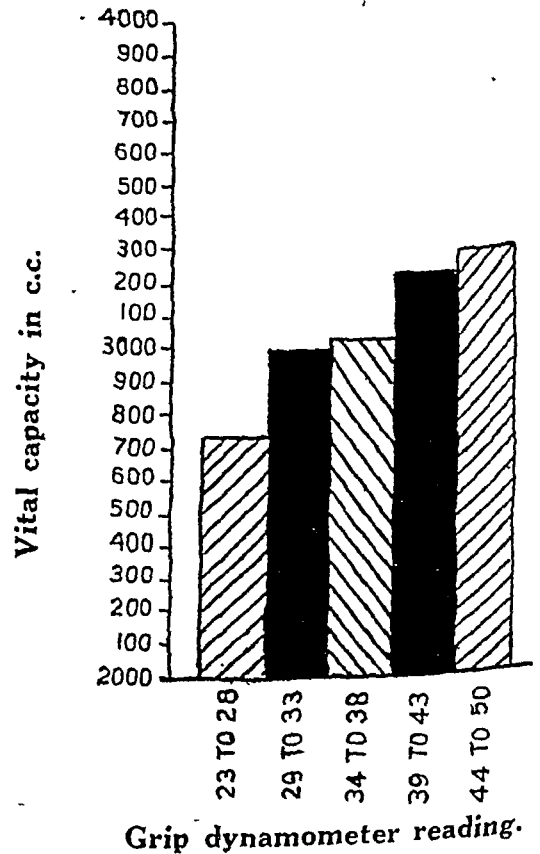
GRAPH 9-b.



GRAPH 10-a.



GRAPH 10-b.



of 17 and 25, the remainder being doctors, teachers and servants. Anatomical measurements (standing height, sitting height, weight, chest circumference, chest expansion, body-surface area, trunk-surface area and chest volume) and also the dynamometer reading for muscle power (hand-grip) were recorded in a large number of them. All measurements could not be obtained in all the subjects. The study includes all classes of people and there was no selection in the subjects. The results obtained are given in Graphs 1 to 10. The number of subjects under each anatomical measurement was not large enough for the mean to be representative throughout each series. So, the readings are also re-grouped into small ranges of anatomical measurements and their mean vital capacity. This re-grouping roughly indicates the nature of distribution of vital capacity among those ranges of anatomical measurements. Both the individual and re-grouped data are represented graphically.

RESULTS.

To measure the vital capacity, Sanborn's spirometer, a wet type marked in c.c., was used. The highest reading obtained in three attempts was recorded.

Total number of subjects	310
Age range	17 to 43 years.
Average vital capacity	2,985 c.c.
Number of students in the group	243
Age range among students	17 to 25 years.
Average vital capacity in students	3,002 c.c.

✓1. *Age and vital capacity.*—

Total number of subjects	219
Range of age in the group	17 to 35 years.
„ „ vital capacity in the group	2,575 c.c. to 3,700 c.c.

Graphs do not show any relationship between age and vital capacity. Results of other Indian workers also show the same absence of relationship.

✓2. *Standing height and vital capacity.*—

Total number of subjects	230
Range of heights in the series	150 cm. to 180 cm.
„ „ vital capacity in the series	2,510 c.c. to 3,850 c.c.
Average standing height	166 cm.
Vital capacity per cm. of height	17.9 c.c.

Graphs indicate correlation between standing height and vital capacity.

✓3. *Sitting height and vital capacity.*—Sitting height was measured between the level of the ischial tuberosities and the top of the head with the subject sitting on a high stool.

Total number of subjects in this series	132
Range of heights in the group	77 cm. to 93 cm.
Average sitting height	85.4 cm.
Range of vital capacity	2,400 c.c. to 3,543 c.c.
Vital capacity per cm. of sitting height	36.7 cm.

The best correlation of all the series is obtained between sitting height and vital capacity.

✓4. *Weight and vital capacity.*—Recorded with the minimum clothing in kilos.

Total number of subjects in the series	226
Range of weights	40 kilos to 76 kilos.
„ „ vital capacity	2,596 c.c. to 3,938 c.c.
Average weight for the group	53.5 kilos.
Vital capacity per kilo	56.3 c.c.

There is good relation between weight and vital capacity as shown by the graph. This is next to relation of chest expansion to vital capacity.

5. *Body surface and vital capacity*.—Recorded in square metres by referring to the charts prepared by Boothby and Sandiford based on Du Bois's formula.

Number of subjects in the group	224
Range of body surface	1.34 sq.m. to 1.92 sq.m.
Average body surface	1.59 sq.m.
Range of vital capacity	2,478 c.c. to 3,850 c.c.
Vital capacity per sq.m. of body surface	1,911 c.c.

There is relation between surface area and vital capacity.

6. *Trunks surface and vital capacity*.—Calculated according to the following formula:—

$$T. S. = A (B + C) \times 0.703 \quad \left\{ \begin{array}{l} A = \text{Distance between jugular notch and symphysis pubis.} \\ B = \text{Girth of chest at nipple level.} \\ C = \text{Girth of abdomen at level of umbilicus.} \end{array} \right.$$

Number of subjects in the series	43
Range of trunk surface (corrected to hundreds)	4,300 sq.cm. to 6,200 sq.cm.
Average trunk surface	5,347 sq.cm.
Range of vital capacity	2,600 c.c. to 3,610 c.c.
Vital capacity per 1,000 sq.cm.	602 c.c.

There is relation between trunk surface and vital capacity.

7. *Normal chest circumference and vital capacity*.—Recorded at the level of xiphisternum at the end of normal expiration.

Number of subjects in the group	217
Range of chest circumference	60 cm. to 100 cm.
„ „ vital capacity	2,450 c.c. to 3,600 c.c.
Average chest circumference	77.5 cm.
Vital capacity per cm. of chest circumference	40 c.c.

From the graph a linear relationship does not seem to exist between chest girth and vital capacity.

8. *Chest volume and vital capacity*.—Measurements are made with anthropometric calipers and calculated according to the formula:—

$$C. V. = XYZ \quad \left\{ \begin{array}{l} X = \text{Length of sternum.} \\ Y = \text{Antero-posterior diameter of chest.} \\ Z = \text{Mid-axillary diameter.} \end{array} \right.$$

Number of subjects	38
Range of chest volume	5,400 c.c. to 9,500 c.c. (correlated to hundreds).
Average chest volume	7,055 c.c.
Range of vital capacity	2,600 c.c. to 3,900 c.c.
Vital capacity per 1,000 c.c. of chest volume	452 c.c.

No linear relationship can be made out.

9. *Chest expansion and vital capacity*.—Recorded at xiphisternal level first, at the end of normal expiration and again at the end of maximum inspiration.

Number of subjects	217
Range of expansion	1 cm. to 10 cm.
Average expansion	5.1 cm.
Range of vital capacity	2,600 c.c. to 3,510 c.c.

Good relation is observed in the graph. It is next to sitting height relation.

10. *Muscle power (hand-grip)*.—Recorded by means of a dynamometer in both hands and maximum reading out of three efforts with each hand taken. The hand-grip is recorded

as this is an index of general muscular build of the individual, and because well-developed respiratory muscles of the chest help its mobility and increase the vital capacity.

Number of subjects	158
Range of dynamometer readings	23 to 50
Average of dynamometer readings	36
" vital capacity	2,567 c.c. to 3,800 c.c.
Vital capacity per one division	86 c.c.

There is linear relationship between grip (muscle power) and vital capacity. When the series are re-grouped, the graph shows the best relation of the series. This indicates that a better relation might be obtained when a larger number of subjects are investigated.

SUMMARY.

1. Vital capacity was measured in 310 male subjects of different sects, classes and ages. The average capacity was 2,985 c.c.

2. Various anatomical measurements such as height, weight, etc., have been correlated with vital capacity. The highest correlation was obtained between sitting height and vital capacity. Chest expansion gave the next best relation. No correlation was found between age and vital capacity.

3. A table showing the results recorded by different Indian workers and English, American, Chinese and Japanese averages has been given (see Table II).

We are thankful to Dr. N. Alahasingari, Professor of Physiology, Andhra Medical College, for his kind guidance and encouragement in this work.

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TABLE I.

Relation between various anatomical measurements and vital capacity.

Anatomical measurement.	Vital capacity per unit.	Average of the series.	Number of subjects in each.
Standing height in cm. ..	17.9 c.c. per cm.	166 cm.	230
Sitting height in cm. ..	36.7 c.c. per cm.	85.4 cm.	132
Weight in kilo. ..	56.3 c.c. per kilo.	53.5 kilo.	226
Body surface in sq.m. ..	1,911 c.c. per sq.m.	1.59 sq.m.	224
Trunk surface ..	602 c.c. per 1,000 sq.cm.	5,317 c.c. (calculated from uncorrected original readings).	43
Chest girth	40 c.c. per cm.	77.5 cm.	217
Chest volume	452 c.c. per 1,000 c.c.	7,055 c.c.	38
Hand-grip (muscle power) ..	86 c.c. per each division	36 divisions.	158

TABLE II.*
Vital capacity figures for Indians.

Investigators.	Number of subjects.	Age in years.	Average vital capacity in c.c.	Vital capacity in c.c. per cm. of standing height.	Vital capacity in c.c. per sq.m. of body surface.	Place from which the results are reported.
1. Bhatia (1929)	100	20-45	3,096	18.52	1,960	Bombay.
2. Telang and Bhagwat (1941) ..	172	18-29	2,949	17.65	1,830	Bombay.
3. Krishnan and Vareed (1932) ..	103	18-29	2,929	17.5	1,850	Madras.
4. Krishnan and Vareed (1933) ..	198	17-26	3,050	18.5	1,930	Madras.
5. Reddy (1933)	105	..	3,156	19.1	1,950	Vizagapatam.
6. Reddy and Sastry (1944) (this paper).	310 (total).	17-43	2,985 (in 310 subjects).	17.9 (in 230 subjects).	1,911 (in 224 subjects).	Vizagapatam.
7. De and De (1939) ..	100	17-23	2,721	16.5	1,790	Bengal.
<i>Indian averages—</i>						
Men (derived from the above data)	1,088	17-43	2,982	17.95	1,889	India.
Women (Mason, 1932) ..	587	16-35	2,150	India.
<i>Chinese—</i>						
†(Foster and Hsieh, 1923) ..	425	Adults	3,180	19.5	2,020	China.
<i>Japanese—</i>						
†(Satake and Sato, 1938) ..	230	19-26	3,800	23.3	2,380	Japan.
<i>English—</i>						
Average	4,342	24.0	2,420	England.
<i>American—</i>						
Average	4,547	26.2	2,540	America.

* This table has been prepared with the help of Table VI in the paper published by D. M. Telang and G. A. Bhagwat, given in the list of references.

† Quoted by Telang and Bhagwat (1941).

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NOTICE.

Scheme for distribution of Penicillin.

1. It is probably now common knowledge that the American authorities have recently agreed to release limited quantities of penicillin for use of the civil population in India and as a corollary they have stipulated that all use of it must be under strict Government control.

2. In order to ensure that the available supplies are equitably distributed throughout this country and reach patients to whom its administration is justified in the current medical practice the distribution and use of penicillin is controlled by the Government of India through committee consisting of five members with the Director-General, Indian Medical Service, as its Chairman. This committee is known as the Central Penicillin Control Board and it is imperative now on every importer of penicillin to comply with any control rules and regulations that may be issued by the Board from time to time. Publication No. 1 of the Board has already been published. This besides giving the dosage and mode of administration of penicillin includes also the diseases in which penicillin is indicated and contra-indicated. Copies of this pamphlet have already been supplied to the heads of the Government Medical Departments in Provinces and States.

3. Naturally the unusual healing properties of penicillin have caught the imagination of the medical profession, so much so that often supply of this drug is requested for the treatment of indefinite cases where everything else has been tried and found to have had no results. One of the objects of the measures of control is to prevent this use of such a valuable drug. Its use is also prohibited in venereal diseases.

4. It is as necessary with penicillin as with sulphonamide, to know the nature of the infection to be treated. While some bacteria are extraordinarily susceptible to it, others are completely unaffected, and to use penicillin in an effort to eliminate them is a complete waste of valuable material. There are three groups of bacteria against which both the sulphonamides and penicillin are effective, with some important individual differences, and these account for most of the infections in which penicillin has been used.

5. Penicillin, in the first instance, will be supplied only to approved institutions, authorized to use this drug by or under the authority of the Board. Any stocks remaining over each month after the various Provinces have been allotted as much as they require will be sold by importers direct to medical practitioners and non-government institutions under the following control:—

Every request for supply of penicillin in such cases must emanate from a medical practitioner and should be accompanied by a certificate that the case for which penicillin is required is suitable for treatment which should be signed by an Administrative Medical Officer, i.e. a Surgeon-General, Inspector-General of Civil Hospitals, Residency or Agency Surgeon, or any of the 'assessors' nominated by him for the purpose. These assessors will ordinarily be surgeons or specialists who have access to a suitable bacteriological laboratory.

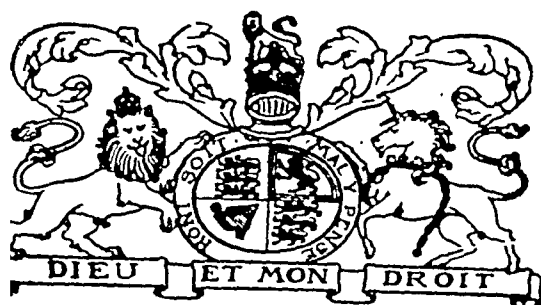
In order to judge whether the use of penicillin is justified the 'assessors' will require particulars of the case which must include the diagnosis of the case, bacteriological findings, the doses, the mode in which penicillin is proposed to be administered and also the quantity required. If the assessors are satisfied they will sign a certificate that the case is suitable for penicillin treatment stating how much penicillin is to be issued. The importer on receipt

of such a certificate will issue the amount of penicillin prescribed thereon. It is incumbent on every medical practitioner or institution obtaining penicillin to submit a case report through the Government Assessor on the prescribed Penicillin Chart copies of which have been distributed to Provincial and State medical authorities.

6. A considerable portion of the quota allotted to India has already reached the hands of the importers. Stocks of penicillin at present are available with Messrs. Kemp & Co. at Bombay, Madras, Calcutta and Delhi. Messrs. Parke, Davis & Co. at Bombay, Madras and Karachi, Messrs. T. M. Thakore & Co., Bombay, Messrs. Martin & Harris Ltd., Calcutta, Messrs. Volkart Brothers. Bombay, Messrs. D. M. Wadia & Co., Bombay, and Messrs. Smith Stanistreet & Co., Calcutta.

—*Editor, I. J. M. R.*

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A CASE OF CANINE *S. TYPHIMURIUM* INFECTION WITH NOTES ON OTHER *SALMONELLA* INFECTIONS IN ANIMALS.

BY

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SINCE Savage (1925) commented on the meagre knowledge regarding animal carriers of salmonella infection transmissible to man it is nowadays difficult for any one but the specialist in this field of research to realize the great extent of new knowledge and to keep pace with it. Thus, 'the number of recognized salmonella types had doubled from the time of the first publication of the Kauffmann-White Scheme in 1934 to the second edition in 1939 and has about trebled by now' (Bornstein, 1943). It is now firmly established that domestic animals are the main source of human salmonella infections: salmonella have frequently been isolated from animal sewage and well and surface water contaminated by it. The problem is further complicated by the fact that certain farm animals, notably swine, occasionally harbour a considerable variety of salmonella without any evidence of active disease. The risk of human infection is further aggravated by the fact that about 2 per cent of rats and mice (America), which are liable to contaminate foodstuffs, carry *S. enteritidis* and *S. typhimurium*, while *S. anatum* and *S. newington* have also been isolated from rats. Of the essentially human types of salmonella, *E. typhosa* has been isolated once from a hen along with *S. pullorum*: mixed salmonella infections are not uncommon in animals. It is worth noting, moreover, that not a few salmonella types isolated from man, and originally considered host specific, have been recently isolated from animals: 'it is highly probable that, sooner or later, these types will appear in septicæmic disease of animals and gastro-enteritis of man' (Edwards and Bruner, 1943). Summarizing this knowledge, these workers catalogue the following types (excluding *S. pullorum* and *S. gallinarum* from domestic fowls) isolated from animal sources according to the frequency of isolation: *S. typhimurium*, *S. cholerae suis* var. *kunzendorf*, *S. typhimurium* var. *copenhagen*, *S. abortus equi*, *S. cholerae suis*, *S. bareilly*, *S. anatum*, *S. derby*, *S. newport*, *S. newington*, *S. senftenberg*, *S. bredeney*, *S. orianenburg*, *S. enteritidis*, *S. dublin*, *S. monteideo*, *S. californica*, *S. new brunswick*, *S. worthington*, *S. kentucky*, *S. minnesota*, *S. munchen*, *S. london* and *S. give*. All these types were isolated from a wide variety of animals, including domestic fowls from which, however, *S. abortus equi*, *S. cholerae suis*, *S. cholerae suis* var. *kunzendorf* and *S. dublin* were not isolated. The following types were recognized: in swine, *S. cholerae suis*, *S. cholerae suis* var. *kunzendorf*, *S. newport*, *S. new brunswick*: in ruminants, *S. typhimurium*, *S. bredeney*, *S. newport*: in horses, *S. typhimurium*, *S. abortus equi*: in foxes and dogs, *S. typhimurium*, *S. cholerae suis*, *S. cholerae suis* var. *kunzendorf*, *S. dublin*.

In India, in spite of the frequency of paratyphoid-like fever in man and the general importance of the salmonella problem in public health work, including milk and meat inspection, the animal salmonella problem has scarcely been touched: the chief reason is the dearth of trained workers and the mass of general animal disease problems that confronts the animal pathologist. Infection of horses due to *S. abortus equi* has been studied in considerable detail (Edwards, Haddow—unpublished work), as horse-breeding is a matter of moment in India. Disease of the domestic fowl due to *S. pullorum* is suspected to exist but the organism has not been isolated. *S. gallinarum* has been isolated in a solitary instance (Cooper and Naik, 1931). From an outbreak of septicæmia in pigeons *S. enteritidis* was isolated (Shirlaw and Iyer, 1937). Septicæmia of calves due to *S. dublin* has been described (Shirlaw, 1935; Rajagopalan, 1938). Manifold (1928) isolated an organism described as *B. ærtrycke* (mutton) from one hound during

The serum of the rabbit used for immunization prior to injection contained no antibodies against *S. enteritidis* (O) when tested in a dilution of 1/5.

This experiment demonstrates the antigenic overlap existing between the strain under investigation and *S. enteritidis*. This overlap is to be expected in view of the antigen formulæ of the 'O' fraction of these organisms, according to Bornstein (*loc. cit.*).

S. typhimurium (I), IV, (V), (XII).

S. enteritidis (I), IX, XII.

() denotes possible absence.

Absorption tests.

(1) 'Dog' (H) group serum, unabsorbed v. 'dog' (H) group suspension	= 250
" " " " v. * <i>S. typhimurium</i> (H) var.	
" binns (H) suspension	= 250
'Dog' (H) group serum, absorbed binns (H) v. binns (H) suspension	= 0
" " " " v. 'dog' (H) group suspension	= 0
binns (H) serum 250 absorbed 'dog' (H) group v. 'dog' (H) group suspension	= 0
binns (H) serum 250 absorbed 'dog' (H) group v. binns (H) suspension	= 0

* *S. typhimurium* var. binns is monophasic in the (H) group phase and is identical with *S. typhimurium* (H) group.

These experiments prove that the 'dog' strain contains the group flagellar factors 1, 2, 3 and is in this respect, therefore, identical with *S. typhimurium*.

(2) 'Dog' (O) serum absorbed <i>S. typhimurium</i> Glasgow (O) v. <i>S. typhimurium</i> Glasgow (O) suspension	= 0
(3) 'Dog' (O) serum absorbed <i>S. typhimurium</i> Glasgow (O) v. 'dog' (O) suspension	= 0
<i>S. typhimurium</i> Glasgow (O) serum (250) absorbed 'dog' (O) v. 'dog' (O) suspension	= 0
<i>S. typhimurium</i> Glasgow (O) serum (250) absorbed 'dog' (O) v. <i>S. typhimurium</i> Glasgow (O) suspension	= 0

These experiments prove that the 'dog' strain contains (O) antigens which are identical with those of *S. typhimurium* Glasgow.

From the above data it will be seen that the organism isolated from the dog is identical, biochemically and serologically, with *S. typhimurium*.

We are indebted to Lieut.-Colonel R. Phease, R.A.M.C., for permission to publish the case.

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A SURVEY OF LATENT DIPHTHERIA IN VIZAGAPATAM.

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INTRODUCTION.

It is well known that clinical diphtheria is less prevalent in the tropics and sub-tropics than in temperate and cold regions. The epidemiology of diphtheria has been extensively studied in many parts of the world, but this subject has received comparatively little attention in tropical countries where diphtheria is not a public health problem of major importance. Although diphtheria is by no means rare in India, relatively little work has been done on its epidemiology. Most urban areas in the tropics are foci of epidemic diphtheria and epidemic outbreaks of greater or less severity are liable to occur at intervals though it is not known whether such outbreaks occur with any regular periodicity. The occurrence of such epidemics has often been observed and it has frequently been reported that they are usually less severe and associated with a lower mortality than those so commonly met with in temperate and cold countries.

An outbreak of diphtheria of more than usual severity occurred in Vizagapatam in 1937 during which most of the cases which occurred were not subjected to bacteriological examination. Of 32 non-hospital cases proved by bacteriological examination only one proved fatal. The incidence of the disease was highest in children between 2 and 15 years of age (25 out of 32 cases) and the remainder occurred in older age-groups among which the oldest victim was 65.

A carrier survey of the local population was commenced in April 1938 and continued until January 1941. The original intention was also to investigate simultaneously the degree of latent immunization of the community by wide-scale Schick testing, but as the necessary reagents could not be obtained in India, this intention had to be abandoned. Bacteriological investigation of children for *C. diphtheriae* was, however, carried out.

MATERIAL AND METHODS.

During the period of survey a total of 707 children between the ages of 2 and 12 years was examined. All the children had reported to the ear, nose and throat outdoor department of King George's Hospital, Vizagapatam. Parents were carefully interrogated for a history of diphtheria and for information regarding conditions of housing and living, but in many cases the information elicited was of doubtful value. Vizagapatam has a population of approximately 70,000, and as children from various parts of the town and from various social strata were included in the survey, the total number examined may be regarded as a fair cross-section of the child population.

Material for examination was obtained by carefully swabbing separately the throat and nose in every case; the ear also was swabbed when discharging from otitis media. Care was taken to swab as large a surface of the naso-pharyngeal mucous membrane as possible. The nasal swabbing was done in every case with the same swab for both the nostrils inserting the swab well into each. These swabs were immediately brought to the laboratory adjoining and used for culture.

Materials on the swabs were directly inoculated separately into a set of one Loeffler's serum slope, one Horgan and Marshall's blood tellurite plate and one tube of Hartley broth. The inoculated media were then incubated at 37°C. In no case was the inoculation delayed for more than one hour. No initial microscopic examination of the swabs was attempted. Ox blood was used in the preparation of the tellurite medium as horse's blood was not available locally. Care also was taken not to employ old samples of potassium tellurite in the preparation of this medium.

TABLE I.

Children between				Male.	Female.
2 and 3 years	21	12
3 " 4 "	27	28
4 " 5 "	40	36
5 " 6 "	48	33
6 " 7 "	43	39
7 " 8 "	67	39
8 " 9 "	54	31
9 " 10 "	42	32
10 " 11 "	36	23
11 " 12 "	39	17
TOTALS				417	290
Total number of children tested				707	
Number of children at or above 5 years				543	
Number of school-going children (day school) above 5 years				{ Male .. 287	
				{ Female .. 127	
					414
History of diphtheria				Nil.	
History of tonsillitis				123	
Tonsils previously removed				18	
				Number tested.	Virulent positive.
					Avirulent positive.
7th April, 1938 to 31st December, 1938				285	3
1939				214	1
1940				198	Nil.
January 1941				10	Nil.

The isolation of diphtheria-like organisms was effected from the tellurite plates forty-eight hours after seeding. When the Loeffler's tube showed the presence of morphological diphtheria bacilli after twenty-four hours, such positives were preserved as reserves to be used when the tellurite plates happened to be either negative for any growth or for morphological diphtheria bacilli. Sub-cultures from tellurite plates were made on Loeffler's medium: From each plate eight different colonies were isolated making eight different cultures from each swab and sixteen from each individual. The growth on the tellurite plate was for some

reason slow and often took more than forty-eight hours for easy recognition and isolation. As the result of test seeding it was also observed that much reliance could not be placed on the colonial characteristics presented on the tellurite plates for the differentiation of types. Within twenty-four hours after isolation morphological characters were studied and what resembled the diphtheria bacillus was inoculated separately into a set of Hiss's serum-water tubes containing one per cent glucose, maltose, saccharose, dextrine, lactose and mannite. The results of these were read after twenty-four and forty-eight hours and recorded. The saccharose fermenters of these were discarded.

RESULTS.

During the survey a total of six strains were isolated which corresponded in their morphological and biochemical characters with *C. diphtheria*. None of these six strains produced any effect on starch or glycogen. All six presented prominent metachromatic granules, gave rise to uniform turbidity in Hartley broth and formed hæmolysin, thus conforming to *mitis* type. Each of the six strains was tested for virulence in guinea-pigs following the technique described by Mackie and McCartney (1938), and four were found to be virulent and two non-virulent. Details of these six strains are given in Table II :—

TABLE II.

Number.	Date.	Site.	Sex.	Age in years.	Glucose.	Maltose.	Saccharose.	Dextrose.	Lactose.	Mannite.	Hæmolysis.	Virulent.
10	13-4-38	Throat	F.	7	Ac.	Ac.	..	Ac.	+	+
12	16-4-38	„	M.	12	Ac.	Ac.	..	Ac.	+	+
162	3-8-38	„	F.	8	A.	Ac.	+	-
187	17-8-38	„	F.	4	Ac.	Ac.	..	Ac.	+	+
251	2-11-38	Nose	M.	10	Ac.	Ac.	..	Ac.	+	-
311	1-2-39	Throat	M.	8	Ac.	Ac.	..	Ac.	+	+

Hæmolysis was tested on 6 per cent rabbit-blood agar. Nos. 162, 251 and 311 were school children.

In the whole series of 707 children examined, only six carriers were detected. All of the six positives were found to be harbouring *C. diphtheria* in the upper respiratory passages, and in five of these this organism was found in the throat. In the throat-negative case, the nasal swab was positive, this being the only occasion on which a positive nasal swab was observed. The organism isolated from the nose was avirulent as was also that isolated from one of the throat positives. It is not known whether these two strains were truly avirulent or merely avirulent variants. In no case was *C. diphtheria* isolated from ear discharge, but the number of cases investigated was very small. The positive children had no history of immediate contact with clinical diphtheria or of previous diphtheria, but the histories were not very reliable. Thus, the latent infection rate with *C. diphtheria* in the child population of Vizagapatam between the ages of 2 and 12 years during the specified time interval works out to be 0.57 per cent virulent and 0.28 per cent avirulent. Topley and Wilson (1936) give 0.6 per cent as the carrier rate among the general non-contact population. Vardon (1923) found a virulent carrier rate of 0.70 per cent in a series of 1,000 persons of all ages drawn from different parts of India and examined at Kasauli.

It is probably significant that three of the four virulent positives were obtained in 1938—the year immediately following an epidemic of unusual severity for Vizagapatani. There was no epidemic worth the name in 1938 and of the 88 swabs from suspected cases tested for the whole year only 7 yielded positive cultures as against the corresponding figures of 283 and 58 in 1937.

In the course of this work the original swabs were also plated on rabbit-blood agar in order to ascertain the infection rate of hæmolytic *Streptococcus* in the naso-pharynx of these children. Out of 200 swabs a positive result was obtained in 14 cases, i.e. 7 per cent. Another interesting observation was an unexpectedly high infection rate with *Staphylococcus*.

In India, epidemics of diphtheria start in a community either from active cases or carriers, as milk is invariably boiled before use. Environmental factors such as the continued close herding of the susceptibles, as would occur in schools, hostels, hospitals and similar institutions, are universally accepted as favouring the rapid diffusion of infection. Such influencing factors are, however, present both in the tropics and in the colder countries. Nevertheless, the disease is relatively infrequent, milder and less fatal in the former. The reason for this difference is not clear. It cannot be fully explained on the ground of infrequent recognition and poorer reporting on the one hand, or on better social conditions, improved methods of treatment or active prophylactic immunization of the susceptibles on the other. Other factors must play a part such as those that influence the organism, those that influence the host and those that influence transmission.

There is ample evidence to show that the biological types of the organism prevalent in a locality would profoundly influence the morbidity and mortality rates and the immunity status occurring in that locality. Marked differences do exist between the diphtheria organisms found in different localities. Radical fluctuations also occur from year to year in their incidence. In any locality there may also occur a sudden replacement of a milder type by a much more virulent one, the *mitis* by the *gravis* type. Whether or not there is any difference between the virulent strains, any peculiar difference in the biological properties encountered in the temperate regions and those in the tropics has not been established. Variations due to the operation of climatic and meteorological conditions cannot be altogether ruled out. But the transmission period from one host to another is so short that it is difficult to envisage any alteration occurring during this period under the influence of such climatic conditions. Besides, there is so far no evidence, based on scientific studies, to support the view that any lowering of virulence is naturally effected by these factors.

The nature of all the defence factors of the body is not well understood. No serious consideration has yet been given to the complex and little known influence of the bacterial flora of the naso-pharynx on the colonization of this area by the virulent diphtheria bacilli. Whether this factor has any rôle in reducing the effective virulence of this pathogen is now only a matter of conjecture.

It is also possible that there may be a high level of endemic immunity developed among the general population as the result of a slow inapparent diffusion of infection favoured by backwardness and insanitation. A favourable host-parasite balance may also play a part and this is supported by the observation that, in places where the immunity status has been assessed by Schick testing, the protection factor is higher in the tropics than among people who live in temperate and cold climates. A high degree of community immunity, probably the result of a high degree of sub-clinical infection, is bound to produce a low case incidence. It is an observed fact that the incidence of clinical diphtheria is relatively less in the tropics than in the higher latitudes. It is true to some extent, as Dudley *et al.* (1934) say, that in coloured races diphtheria infection tends to be more concentrated in the lowest age-groups than is customary among Europeans and so cases that do occur rarely attract the attention of medical men. Experience does not support this suggestion as the sole explanation, and it is doubtful whether racial peculiarities are in any way responsible. There is no scientific basis for the assumption that the biological response of the tissues of coloured peoples to infection differs in any way from that of others. Environmental conditions, by favouring greater opportunities of close contact between man and infection, are probably much more important factors. The system of boarding schools and dormitories is admittedly uncommon

in the tropics, but the low economic state and the peculiar social conditions of tropical populations produce similar environmental conditions at an even earlier age-period and in a more exaggerated form. Immunity, therefore, would tend to develop earlier in life in the tropics than in other parts of the world. The experience of Pasricha *et al.* (1939), limited as it is, tends to support this view. The incidence of the disease and of the carrier state are both the outcome of the interplay of many factors and no single factor can be held solely responsible for these.

SUMMARY.

1. An account of an investigation into the carrier rate of *C. diphtheriae* in Vizagapatam has been given.

2. Swabs from the throat and nose of 707 children between 2 and 12 years of age were examined over a period of nearly three years.

3. *C. diphtheriae* was isolated from six of these children in none of whom were history or symptoms of diphtheria present. Four of the six strains were found to be virulent for guinea-pigs and two avirulent. The 'virulent carrier' rate for this locality was found to be 6.57 per cent and the 'avirulent carrier' rate 0.28 per cent.

With great pleasure I record my thanks to Professor H. B. Maitland of Manchester University and to Professor C. Ramamurty for their helpful suggestions, to Dr. N. V. Subrahmanyam of this Department, to Dr. B. Tirumal Rao, the E. N. T. Surgeon of the K. G. Hospital, Vizagapatam, and Dr. M. N. Prabhu of the same Department for their very valuable co-operation and help, and to Dr. D. Subba Rao, the local Municipal Health Officer.

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ÆTIOLOGY AND TREATMENT OF *ULCUS TROPICUM*.

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Ulcus tropicum was extremely rare in Bengal until the year 1943 when, during the months of July to December, a large number of cases occurred in Calcutta. Reports were also received at the time that cases of *Ulcus tropicum* were occurring in epidemic form in other places in Bengal, Orissa, Santhal Perganas and Nagpur.

There is an extensive literature on tropical ulcer, relating chiefly to its clinical description, predisposing causes, diagnosis and treatment but few regarding its ætiology. The writer's first attempts to investigate the ætiology of the disease began in 1932 and the results of this earlier work were published in the *Annual Report of the Calcutta School of Tropical Medicine* (Acton, 1932). Results of later work were published in the *Transactions of the Calcutta Medical College Re-Union* (Panja, G., 1937-38). A suggestion was made there that *fusiform* bacilli implanted on injured or diseased skin caused the disease, but no conclusive evidence had been obtained.

During the investigation of a large number of cases in Calcutta in 1943 the following special clinical features were noted with regard to ætiology of tropical ulcer :—

1. The ulcers were almost invariably confined to the lower legs, but in two out of 58 cases, the front aspect of one of the knees was involved. This suggests that the infection, if there be any, is derived from the soil.

2. The gums were found healthy in most cases. The possibility of infection with *fusiform* bacilli from gums, as reported by Apostolides (1922) and Clements (1936) was remote.

3. There was no observable local or general immunity in the disease. A fresh sore could be reproduced at the site of a healed ulcer by inoculating the pus from an active sore. One patient after being cured of multiple ulcers, returned with two big ulcers, after two months. In some cases, a fresh sore broke out at a different site when an old sore was nearly healed.

These facts suggest that the ulcer is not caused by a virus which generally gives a solid long-standing immunity.

Predisposing causes and vectors.

1. Most of the cases examined were bare-footed Indians belonging to the middle and poorer classes. This again suggested that the infection was derived from the soil.

2. The ulcers generally started at the site of injury to the skin or other lesions such as impetigo or boil as reported by other workers. Many cases received injuries from shoes and tramples while waiting in a crowd to buy food. A suggestion is therefore put forward that damaged and devitalized tissue may predispose to the development of tropical ulcers. There was one interesting case of a boy who received an injury in a military contractor's firm with tools used and sent back from the Assam frontier. It is well known that *Ulcus tropicum* is prevalent in Assam and it is possible that the infection was carried on the tools.

3. General health was poor in some cases on account of starvation but some patients were in excellent health, there being no signs and symptoms of any vitamin-deficiency. The theory of deficiency in vitamins has been put forward as the direct cause of the disease but this cannot explain why ulcers should occur on the lower legs only. The theory of malnutrition was also negatived by Hughes (1931) who showed that there was no improvement by feeding with cod-liver oil, orange juice and yeasts. Buchanan and Sanderson (1935) also found no response to diet rich in vitamins. It is possible that deficiency may be a predisposing cause and this point has been stressed by Clements (*loc. cit.*). No peripheral neuritis was found in any case as reported by Napier (1943).

4. Flies and termites have been reported as probable vectors of infection by Fox (1920-21) and Young (1932). No evidence in support of this contention was obtained in the present investigation.

It is possible that the war has favoured the spread of the disease from Assam to Bengal and other provinces by evacuation of infected persons, carriage of infected soil by increased motor lorry transport and partly possibly also by helping to create more prevalent malnutrition and consequent enhanced susceptibility.

BACTERIOLOGY.

1. *Fusiform bacilli*.—Gram-positive *fusiform* bacilli were found in the pus of all typical ulcers in the active stage, and the presence of such *fusiform* bacilli came to be regarded as a diagnostic picture. In none of the cases studied in this or the previous series were *fusiform* bacilli absent when the ulcers were typical and active, i.e. when thick greyish pus, acute

tenderness and a raised shelving margin were present. Although the bacilli were Gram-positive, they became Gram-negative after prolonged decolorization with alcohol. This fact is important, as all cultures of *fusiform* bacilli were Gram-negative.

The *fusiform* bacilli tended to disappear when the ulcers were healing but reappeared when healing was interfered with by artificial means. There was a constant association between the presence of *fusiform* bacilli and the characters of a typical tropical ulcer but no such association was observed in the case of other micro-organisms sometimes found in the ulcers.

A fact of great importance is that, in some ulcers, *fusiform* bacilli only were found on repeated microscopical examination and that no other organisms were detected by aerobic culture on blood-agar.

In an unopened pustule in the neighbourhood of a tropical ulcer, no *fusiform* bacilli were found but *Staphylococcus* and *Streptococcus* were isolated. This pustule gradually assumed the characters of a tropical ulcer when it was broken and contaminated with the pus from the neighbouring tropical ulcer.

Histological examination of ulcerous tissue showed *fusiform* bacilli in the granulation tissue, arranged in a palisade from which penetrating chains invaded the deeper parts. Cocci and other organisms, if present, were above the row of *fusiform* bacilli. The above facts suggest that *fusiform* bacilli may be the cause of the disease.

2. *Vincent's spirochaetes* were seen in some ulcers but were absent in the majority. A careful search was made in material stained with Gram's, Leishman's and Giemsa's stains including the serum exuded after scraping but in most cases none were found. Absence of spirochaetes has also been reported by Wollbach and Todd (1912-13), Fox (*loc. cit.*), Corpus (1924), James (1938) and Basu (1913).

3. *Other micro-organisms*.—Gram-positive cocci chiefly *Streptococcus pyogenes* were found in some ulcers. *Staphylococcus aureus*, diphtheroids, Gram-negative bacilli and occasionally *Clostridium* were also found but there was no constant association between any particular organism and the active stage of an ulcer. In fact, in some ulcers cocci were found even up to the healing stage. No typical ulcers could be reproduced on human volunteers with cultures of any of these organisms.

4. *Virus*.—Pus from tropical ulcers from seven cases filtered through L₃ porcelain candles and inoculated into the chorio-allantoic membrane of developing hen's eggs did not reveal the presence of any virus on incubation.

Cultivation of the fusiform bacillus.

The organism was successfully cultivated on sheep-blood agar in which a little sterilized pus from tropical sore was incorporated. A thick inoculum was stroked on a blood-agar slope or plate which was incubated under anaerobic conditions at 37°C. As blood-agar plates were often found contaminated after 3 to 4 days' incubation it was found more suitable to use blood-agar slopes with cotton plugs. Pyrogallic acid powder and 10 per cent caustic potash were put on the top of the plugs and this was immediately followed by the application of tight rubber-caps. Only two longitudinal strokes were made with a loopful of thick pus collected from the bottom of an ulcer. Fine translucent colonies were seen after 3 to 4 days' incubation at 37°C. Any fine colonies which appeared within 24 hours were never due to *fusiform* bacilli but usually to *Streptococcus*. Later as a routine, cultures were taken on two or more 'pus-blood' agar slopes, one on Loeffler's serum and one aerobically on ordinary blood-agar in order to see whether there were any aerobic organisms in the ulcer pus. If there was no growth on aerobic culture, *fusiform* bacilli were more commonly isolated in pure culture under anaerobiasis.

On sub-culture, sometimes fine and coarse types of colonies were found. Sometimes mucoid colonies were seen. Colonies were low convex with an entire margin but sometimes older colonies showed a crenated margin with a central depression or elevation. It was difficult to maintain a culture. The majority were viable up to 7 to 10 days only but a few remained alive up to 26 days. Hence repeated sub-cultures were necessary.

Morphology.—The organism was Gram-negative, *fusiform*, arranged either singly or in pairs or in chains characteristically clustered. Thickenings were found sometimes in the middle or at one end of the bacilli. These were not spores as these were not found free and did not take any spore-staining. In a few cultures a mixture of spirillar forms and *fusiforms* was found as was seen also in some specimens of pus. In one culture, pure spirillar forms only were found and the colonies were very fine and visible with a hand lens only but on repeated sub-culture usual colony forms and *fusiforms* were seen. The spirillar forms seen did not resemble Vincent's spirochaetes.

The bacilli were motile showing brisk and twisting movements. Sometimes there was circling movement. Motility was also visible in pus films.

As the organism was Gram-negative in culture and found often in chains, there was some doubt at first whether true *fusiform* bacilli were isolated. But this doubt was dispelled when it was found that at least some of them were *fusiform* and some were feebly Gram-positive. Pure cultures of such organisms were obtained from samples of pus which, on repeated microscopical examination, had shown *fusiform* bacilli only. *Ulcer tropicum* was reproduced experimentally with Gram-negative bacilli from such cultures and in films from the experimental sores, Gram-positive *fusiform* bacilli only and no other micro-organisms were demonstrated.

Attempts at cultivation of the *fusiform* bacilli on various other media were made but failed. Trials from a few cases were made on horse-serum agar (Weaver and Tunncliffe, 1905), ascitic-agar slants (Tunncliffe, 1906 ; 1911), Dorset's egg (Peters, 1911), blood-agar and potato-extract agar with gentian violet (Slanetz and Rettger, 1933) and also various other media but were of no avail. Smith's (1933) technique in sheep-serum agar with a fragment of ulcerous tissue was tried twice and Krumwiede and Pratt's (1913) method of anaerobiasis was followed but without success. As the technique was not simple and difficulty was experienced in detecting and picking off *fusiform* bacilli from cultures containing other facultative anaerobic micro-organisms, further trial was dropped. The method and medium adopted as a routine were simple, a large number of cultures could be put up within a short time and examination of colonies and sub-cultures could be made easily without damaging the medium. The only disadvantage was that cultures were not successful in every case on account of heavy contamination with secondary invaders. This was largely obviated by dressing the cases for one or two days with sulphathiazole dressing powder and then with sterile saline. When on aerobic culture few or no micro-organisms were seen and still *fusiform* bacilli were abundant in films, cultures were taken on several 'pus-blood' agar slants.

Experimental production of sore.

Infectivity of the pus from tropical ulcers was first tested. An intradermal inoculation of a small amount of pus into arms or legs of man often led to the development of a typical ulcer showing *fusiform* bacilli in 3 to 6 days. Animals, such as monkeys, dogs, rabbits, guinea-pigs, white rats, mice and pigeons were found to be refractory. In some animals, abscess or atypical sores developed but *fusiform* bacilli were never found in any of the lesions. Nigerian hedgehogs were not available and therefore Smith's (1936) observation could not be repeated.

Monkeys and white rats fed for one month with autoclaved polished rice, were found insusceptible to inoculation either with the pus from ulcers or pure cultures of *fusiform* bacilli. Pus or cultures of *fusiform* bacilli mixed with ionizable calcium salts was also tried but no positive result was obtained.

Eleven persons were inoculated intradermally either on the upper arm or lower leg with 0.2 c.c. of a thick suspension of five different strains of pure cultures of *fusiform* bacilli in the 3rd or 4th generation. In all cases sores developed at the site of inoculation, varying from $\frac{1}{2}$ " to 1" in diameter within 4 to 6 days. Fever with rigor, oedema, redness at the sites of injection and sometimes generalized oedema of the whole limb were present. Such marked reactions were probably due to the heavy dosage used but these soon subsided leaving only an ulcer discharging copious pus and showing typical Gram-positive *fusiform* bacilli in films and cultures but no spirochaetes. Ulceration was preceded by the appearance of a purulent bleb and marked tenderness was present. There was great difficulty in getting further volunteers

for inoculation experiments on account of the pain experienced. Experimental success was more often achieved when a bandage was applied and maintained for 2 to 3 days after inoculation and the site of inoculation was squeezed with a pair of sterilized forceps immediately after inoculation and on two subsequent days so as to crush the tissue and produce the required O-R potential for multiplication of the *fusiform* bacilli. Ulcers healed up in 10 to 20 days without any treatment excepting that a sterile dressing was kept on. Cultures from artificial sores yielded pure growth of *fusiform* bacilli and with sub-cultures from experimental sores further tropical ulcers were reproduced. (Ulcers reproduced with a mixture of *fusiform* bacilli and *Streptococci* were bigger and took a longer time to heal.)

In a leper suffering from multiple trophic sores near each other on one of his legs a culture of *fusiform* bacilli was applied by scarification on one of the sores and a bandage was applied so as to exclude outside contamination and favour anaerobiosis. The typical characters of tropical ulcer developed in a few days and *fusiform* bacilli were found but other sores in the neighbourhood were unaffected.

Pus from tropical ulcer showing *fusiform* bacilli only was inoculated into some ordinary ulcers and a bandage was applied. The characters of a typical tropical ulcer developed in a few days and *fusiform* bacilli were found in the pus.

In a few persons inoculation either with the pus from an ulcer or pure culture of *fusiform* bacilli was not successful.

Small doses of cultures of *Staphylococcus aureus*, *Streptococcus pyogenes*, diphtheroids and *Pseudomonas pyocyanea* that were isolated from some ulcers were inoculated intradermally into volunteers, followed by crushing of the tissues at the site of inoculation but no typical tropical ulcer with presence of *fusiform* bacilli could be reproduced.

In one case, besides the *fusiform* bacilli, anaerobic spore-forming organisms belonging to the genus *Clostridium* were isolated. These on inoculation into volunteers gave rise to fever, oedema at the sites of inoculation and ulcers similar to tropical ulcers, discharging copious pus full of Gram-positive bacilli. But these bacilli were not like *fusiform* bacilli in appearance and unlike *fusiform* bacilli produced changes in cooked meat media.

Filtrates of pus from tropical ulcer passed through L₃ candles were found non-infective and no ulcer could be reproduced by inoculation of such filtrates.

All the above findings are consistent with the belief that the *fusiform* bacillus is the true cause of *Ulcus tropicum*. The evidence in favour of this theory is summarized below:—

1. *Fusiform* bacilli are always found in active sores, even when the sores are followed up from day to day. It is only when the ulcers are healing and covered with red granulation tissue that no bacilli are found. But the Gram-positive cocci which have been described by Roy (1928) as being the probable cause are present even in the healing stage and sores reproduced by such cocci show neither typical characters of tropical ulcer nor *fusiform* bacilli in the pus.

2. On histological examination of an ulcer *fusiform* bacilli are found penetrating into the deeper layers of the granulation tissue, while secondary invaders are found more superficially.

3. Pus showing only *fusiform* bacilli by microscopical as well as cultural examination was found infective but the filtrate of such pus was found non-infective.

4. In seventeen sores from different cases *fusiform* bacilli only were found by anaerobic culture and no organisms were isolated by aerobic culture. In eight of these sores, repeated examinations were done.

5. The disease was reproduced in eleven volunteers with the 3rd or 4th sub-culture of the organism and the organism was recovered in pure culture from experimental sores. Tropical ulcers were also reproduced with cultures of *fusiform* bacilli from experimental ulcers. Heavy inoculum, intradermal injection, crushing of tissues after inoculation and application of bandage so as to prevent access of air and light were the keynotes of success.

6. *Fusiform* bacilli isolated from all ulcers were agglutinable with the sera raised against several strains of the bacilli and agglutinins were found in some of the case sera during recovery. This experiment could not be fully carried out as the bacilli themselves were found auto-agglutinable.

That the *fusiform* bacillus may be the cause of tropical ulcer was suggested by some previous workers and Brown's (1935) important observation and remarks may be quoted here. 'It must be admitted that it is far from easy to dismiss these organisms (*fusiform* bacilli) as saprophytes and the tendency is to suspect them as being causal, yet a complete chain of evidence has never been established. It is felt by the writer that they are causal as they are almost constantly associated with a definite clinical condition whereas, were they saprophytic, they would be constant inhabitants of all ulcers.'

PLATE I.



FIG. 1.—Experimental reproduction of tropical ulcer with pure culture of *fusiform* bacilli.



FIG. 2.—Experimental reproduction of sore with pure culture of *fusiform* bacilli.



FIG. 3.—*Fusiform* bacilli as seen in Loeffler's serum culture

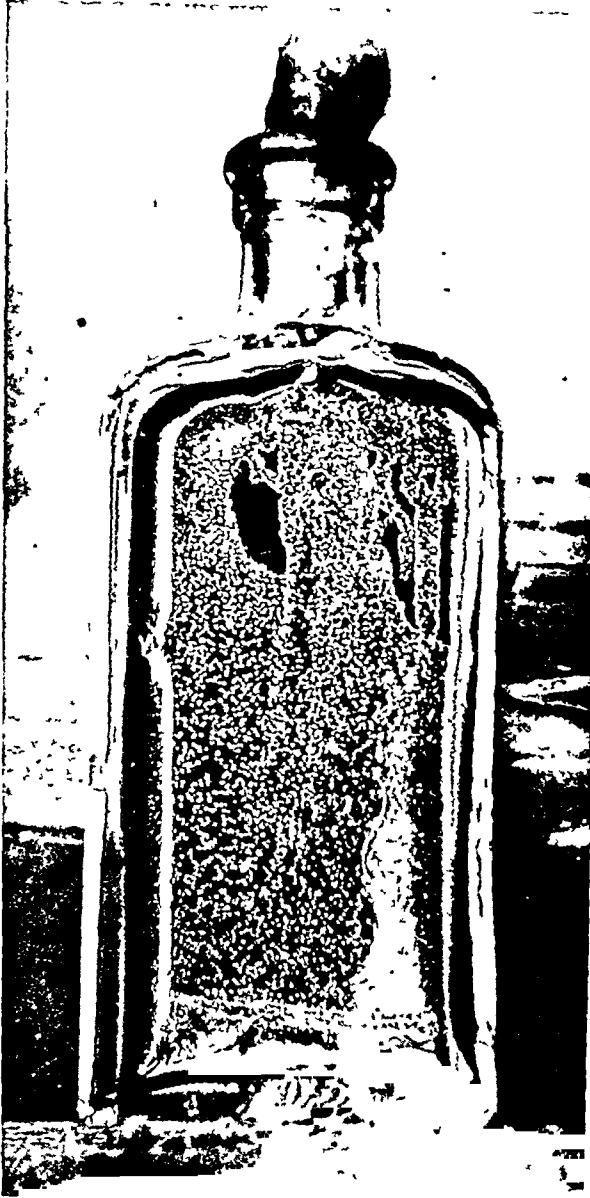


FIG. 4.—Colonies of *fusiform* bacilli, natural size, 3 to 4 days' old.

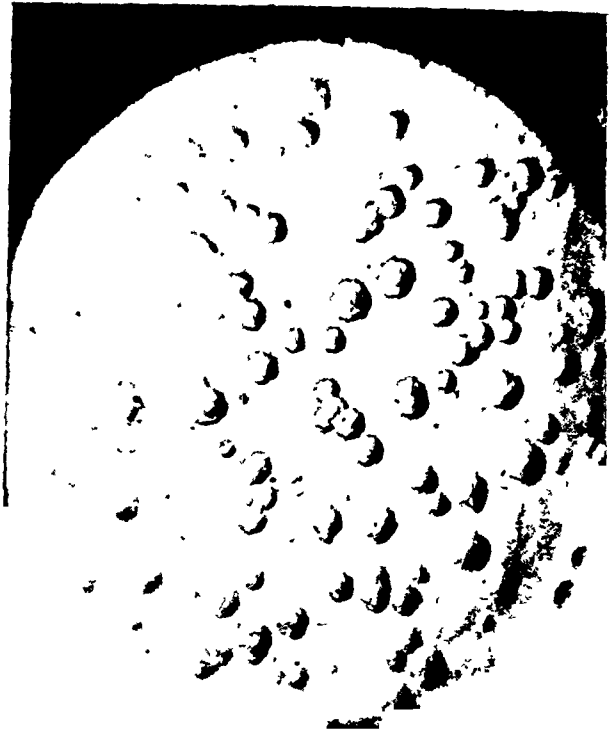


FIG. 5.—Colonies of *fusiform* bacilli, magnified, 3 to 4 days' old.



FIG. 6.—*Fusiform* bacilli in clustered chains as seen in 'pus-blood' agar culture.

'Smith believed the Spironema to be the causal organism...but the culture was not pure. His results were not confirmed by the writer.'

'The whole problem is one of great difficulty and nothing will ever be completely established until some worker succeeds in obtaining a pure culture and a fair number of human volunteers for inoculation.'

Experimental tropical ulcers were reproduced by inoculating pus (Blanchard, 1914; Balliano, 1916; Fox, *loc. cit.*; Smith, 1936) but no previous worker appears to have established the causal organism by reproducing the sore with a pure culture. Fox (*loc. cit.*) in India inoculated with *impure* cultures of *fusiform* bacilli and his experimental sores were atypical, 'not at all like Naga sores' in his own words. This worker has not recorded the result of examination of pus from experimental sores. Smith (1933), in Africa, was the first person to try inoculation experiments on eight volunteers with a pure culture of *fusiform* bacilli isolated by him. Boil-like lesions resulted in only two of the volunteers and typical ulceration was not observed; films showed only scanty *fusiform* bacilli and the ulcers regressed in two to five days.

Dietetic cause.—The influence of diet in the causation of *Ulcus tropicum* has been stressed by several members. The author's observations indicate that diet is not a factor of importance for the following reasons:—

1. The disease was not seen in Calcutta prior to the recent epidemic although dietetic deficiency has long been common.
2. The disease was seasonal and the epidemic subsided in spite of the dietetic deficiency being continued.
3. Ulcers occurred in persons with no signs of diet deficiency.
4. In a family when one member was affected, others were rarely affected although the same dietetic conditions prevailed.
5. Ulcers were almost invariably limited to the lower legs.
6. Healthy volunteers were successfully inoculated with ulcer material or cultures of *fusiform* bacilli.
7. Ulcers responded well to copper sulphate treatment but not to diet supplements.

Treatment.—The cheapest and most effective form of treatment consisted in repeated dressing, at least three times a day, so as to remove the anærobic condition present in the thick layer of pus over the ulcers, favourable for the development of the anærobic *fusiform* bacilli. The ulcers were irrigated either with plain boiled water or weak mercuric chloride lotion and while the jet of an irrigator was playing, all adherent thick pus was disengaged with a sterile cotton swab. The following lotion modified from the formulæ of McGuire (1933) and James (*loc. cit.*) was then painted on, and this was followed by irrigation to remove excess of copper sulphate and to minimize the pain caused by the paint:—

Copper sulphate	2 dr.
Phenol	1 dr.
Glycerine	1 oz.

Finally, a thick absorbent pad soaked in a solution of potassium permanganate (1 in 5,000) was applied. Under this treatment, improvement was seen in two to four days in all cases.

Various other local remedies such as acriflavin, gentian violet, picric acid, mercuric chloride lotion (1 in 1,000) were tried without much success. Sulphonamides have been reported to be efficacious (Bharucha, 1943; Panja, D., and Ghosh, 1944) in tropical ulcers but in the author's experience they were found of little value whether given by the mouth or applied directly to the ulcers as a dusting powder.

A stock vaccine prepared from cultures of *fusiform* bacilli was found to be of no therapeutic value.

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INVESTIGATIONS OF GROUND-WATER POLLUTION.*

Part II.

SOIL CHARACTERISTICS IN WEST BENGAL, INDIA, AT THE SITE OF GROUND-WATER POLLUTION INVESTIGATIONS.

BY

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In a previous paper (Dyer and Bhaskaran, 1943) we reported the results of analyses of soil samples from a site in West Bengal chosen for studies in the flow of pollution in the ground water from a seeded latrine. These samples were obtained when the wells were initially driven and were limited to the area below the latrine in the direction of flow. As the experiment progressed it was felt that the soil data secured might not be representative of the entire area, and the following additional investigation was carried out. Successive soil samples were collected at 2-foot depths from the surface down to 20 feet. Sampling sites were randomly distributed over the entire experimental area, and at least one sample was collected in every zone.

Since mechanical and physical properties and homogeneity of the different layers of soil determine the efficiency of the filtration process, pollution flow under different conditions should be considered with reference to these fundamental properties of the soil before any general application of the results to all soil conditions is attempted.

Chemical examination of the soil made at different levels will show the presence of chalk, which has an important effect upon the underground flow of pollution. A knowledge of other chemical constituents of the soil that are likely to affect the ground water are also important in interpreting flow of chemical pollution. The viability of *B. coli* and other pathogens in the ground water are dependent upon soil conditions, especially its organic matter content.

Investigations in the Punjab (Dyer, 1939) showed that a knowledge of the lactose-fermenting organisms in the soil was necessary in interpreting the bacteriological changes in ground water. Study of the nature and distribution of these organisms should reveal the presence of any underground pockets of pollution in the experimental area.

A rather detailed study of the experimental site at Singur in West Bengal was therefore undertaken, and the results are discussed in the present paper.

EXPERIMENTAL PROCEDURE AND RESULTS.

Sampling procedure.

Satisfactory sampling to a depth of 8 feet could be carried out by using the earth-auger, but below this depth the soil from the upper layers caved and became mixed with the samples from the lower ones. Protecting casings were therefore inserted in the holes, and sampling below 8 feet was done with a sludger. A few preliminary tests showed that this procedure yielded satisfactory results. After collection, the samples were well mixed and air dried in

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thin layers. When thoroughly dried, the lumps were broken, and representative portions of each sample were used for analysis.

Physical properties of the soil.

The soil which was removed while making borings for the installation of the wells at the experimental site was examined for 'kankars' (lumps of impure CaCO_3 common in the soil) and pebbles. No extra borings were made, as there was a large number of representative samples for examination. The results showed that except for the sporadic occurrence of a few pebbles—not more than 10 at a time from any one bore—the soil in the experimental area was free from kankars to a depth of 25 feet. A certain amount of gravel was encountered in the neighbourhood of one of the observation wells (C_2) and in the bore-hole latrine below the 25-foot depth, but it persisted for only a few inches.

The mechanical composition, effective size, and uniformity coefficient were determined for the 70 samples. For those low in clay, the sieve method described by Hazen (1920) was used, and for samples high in clay the U. S. Bureau of Soils procedure (Robinson, 1930) was used. The results are summarized in Table I.

The soil at the experimental site was a sandy loam typical of alluvial deposits. The surface contained about 20 per cent of clay, which decreased with increase of depth up to 16 feet, when only sand remained. Effective size and uniformity coefficients show that the water-bearing sands were quite homogeneous, with properties approaching those of the sand-bed of a slow sand-filter.

The soil samples below 14 feet were examined for coefficient of permeability, porosity, and capillary force. The procedure followed was the same as that adopted by the Irrigation Research Institute, Punjab (Vaidhinathan and Luthra, 1934), and the data are summarized in Table II:—

TABLE II.

Physical properties of soil samples below 14 feet.

MEAN OF 7 SAMPLES AT EACH DEPTH.			
Depth in feet.	Permeability coefficient (cm./sec.).	Capillary force (dynes per sq. cm.).	Porosity (per cent).
14-16	0.058±0.01	2.66±0.19	44.0±0.61
16-18	0.063	2.43	43.0
18-20	0.080	2.40	41.3

The results show that differences in physical properties of the different layers were not pronounced and that, for all practical purposes, the soil was homogeneous.

The continuity of the different layers and the homogeneity of the soil-formation in the experimental area are shown in Table III. Finer particles were present in considerable amounts to a depth of 14 feet, and their distribution varied somewhat from site to site. Below 14 feet the medium was made up mainly of coarse and medium sand and, for all practical purposes, may be considered homogeneous.

Data in Table III were tested by variance. The composition of the soil from site to site was remarkably similar, but that of successive strata at all sites differed significantly.

TABLE III.

*Percentage distribution of fine particles and of sand in the soil.**Fine particles (very fine sand, silt, clay).*

Depth in feet.	SAMPLING SITE IN FEET.						
	BHL-5	5-10	10-15	15-25	25-35	35-50	50-65
0-2	95.3	95.7	89.4	90.2	45.5	48.7	91.4
2-4	81.3	87.4	87.5	81.6	49.7	83.7	85.8
4-6	81.1	62.1	87.5	..	83.6	77.1	83.2
6-8	65.8	..	77.8	82.8	49.7	45.4	33.7
8-10	39.7	51.7	58.6	85.2	51.2	51.3	18.8
10-12	82.6	44.7	35.5	37.9	14.0	39.7	22.4
12-14	49.0	30.8	24.0	19.6	..	21.3	9.6
14-16	4.0	17.5	10.5	9.5	6.5	7.0	4.0
16-18	1.5	3.0	8.0	5.0	4.0	5.5	2.0
18-20	1.5	1.5	1.0	1.2	1.0	3.2	2.5

Coarse and medium sand (below 14 feet).

14-16	71.5	53.0	59.0	70.5	75.2	75.5	81.5
16-18	79.5	81.0	65.5	78.5	80.0	74.0	87.5
18-20	77.0	86.5	88.0	90.0	90.0	77.5	79.0

Chemical properties of the soil.

The chemical analysis of the soil samples included estimations of organic matter, different forms of water-soluble nitrogen, carbonates, sulphates, chlorides, and the hydrogen ion concentration (pH). Total soluble minerals that are likely to affect the conductivity of ground water were also determined by shaking the soil samples with distilled water (25 g. with 250 c.c. of water) and testing the supernatant liquid. The pH was determined with a glass-electrode outfit. Humus and total nitrogen were determined by the method described by Bhaskaran *et al.* (1936). The other constituents were estimated according to procedures outlined by the Association of Official Agricultural Chemists (1935). Average values based on 9 samples are given in Table IV:—

TABLE IV.

Chemical analysis of soil.
(Results expressed on air-dry basis.)

Depth in feet.	MEAN OF 9 SAMPLES AT EACH DEPTH.									
	pH.	Total soluble salts (electrical conduc- tivity).	Carbonates (per cent CaCO ₃).	Chlorides (p.p.h.t. NaCl).	Sulphates (per cent Na ₂ SO ₄).	Humus (per cent organic carbon).	Total nitrogen (per cent).	Free ammonia (p.p.m. NH ₃).	Albuminoid ammo- nia (p.p.m. NH ₃).	Nitrates (p.p.m. NO ₃).
0-2	6.6	35	0.02	1.05	Trace	0.232	0.028	2.72	18.1	0.05
2-6	6.7	21	Nil.	1.02	..	0.156	0.021	1.78	17.1	0.04
6-10	6.8	20	0.06	0.99	..	0.148	0.019	2.78	18.3	0.03
10-14	6.3	26	0.05	0.98	..	0.283	0.028	13.41	14.9	0.04
14-18	6.6	21	0.01	0.96	..	0.076	0.007	16.71	7.0	0.04
18-20	6.7	18	0.01	1.02	..	0.051	0.004	9.00	5.6	0.05

The soil was slightly acidic in reaction and well buffered by the presence of bicarbonates. It contained little organic matter mainly of vegetable origin and this was practically absent below 14 feet. Large amounts of ammonia were present especially in samples below 14 feet. The amount of total soluble minerals was not large when compared with the conductivity of the ground water, and those present could be accounted for by the chlorides, carbonates, and nitrates. The low carbonate content in the samples showed the absence of chalk in the experimental area.

Scrutiny of the results of the chemical analysis of the different layers of soil collected in different zones (Table IV), shows no concentration of any of the chemical constituents at specific sites or depths of the soil.

Bacteriological properties of the soil.

Examination of the soil for presence of bacteria below the water table is never very satisfactory, and the following additional precautions were therefore adopted to prevent contamination, as far as possible. A 2-inch earth-auger was used for collecting the samples. Before each charge, the boring end of the auger was sterilized by flaming. After removal from the bore the inner core of soil was transferred to a sterile basin and was thoroughly mixed, under sterile conditions. Representative portions of the mixed soil were then transferred to sterile bottles, and the wet samples were inoculated in broth tubes within 4 hours of collection.

Since the samples contained varying amounts of moisture, parallel aliquots of each sample were simultaneously weighed out for moisture determination, so that the bacteriological results might be calculated on a comparable moisture-free basis.

Bacteriological analysis included determination of the nature and concentration of gas-formers in MacConkey broth at 37°C. The soil samples were diluted in sterile tap-water, and suitable quantities were inoculated in double strength MacConkey broth tubes. From the number of tubes showing acid and gas in 48 hours the most probable number of gas-formers was calculated according to the procedure described by Thomas (1942). The gas-formers were then plated out and further differentiated by the indol, methyl red, Voges-Proskauer, and sodium-citrate tests. Twenty-one soil samples collected at different depths at 3 sites were analysed, and the results are summarized in Table V:—

TABLE V.

Most probable number of gas-formers in 1 g. of soil.
(Expressed on moisture-free basis.)

Place of collection.	DEPTH IN FEET.						
	2	4	6	10	14	18	20
Between 5' and 10'	41	42	42	8	11	269	23
Between 15' and 25'	29	269	*	3	3	1	8
Between 25' and 35'	8	*	14	15	*	1	*

* Indicates less than 1.

In view of the unsatisfactory sampling procedure below the water table, much reliance cannot be placed on the most probable number in the different layers. The results at least show that gas-formers were present in large numbers in the soil. The differential test of the gas-formers indicated the complete absence of *B. coli*. Of the 11 cultures studied, 9 were *aerogenes* type 1, and the other 2 were citrate-positive soil bacteria.

DISCUSSION.

The marked homogeneity of the soil at different depths in the water-bearing region, as revealed by the analysis of mechanical properties, is an important factor in judging the flow of pollution. For all practical purposes, the soil occurring below 14 feet may be considered a homogeneous filter-medium made up almost entirely of sand. The maximum rate of lateral percolation through the medium under the conditions of this experiment may be estimated by applying Darcey's formula. For a ground slope of 1 in 200 and a coefficient of permeability of 190 feet per day (Dyer and Bhaskaran, 1943), about a gallon per square foot per day may flow through the soil at depths below 14 feet. It may also be observed, in this connection, that this rate is less than the demand flow for efficient filtration, in a slow sand-filter.

Chemical analysis showed that, with the exception of ammonia, the soil constituents could not appreciably affect the ground-water composition. No pockets of chemical constituents or chalk were observed in the soil. Low content of organic matter indicates that conditions below 14 feet were not favourable for the growth of *B. coli* or other pathogens.

Bacteriological sampling of soil below the water table is not satisfactory, so that much reliance cannot be placed upon the most probable number of gas-formers in the deeper regions; nevertheless, the results show that gas-forming bacteria were present in large numbers in the soil but were either aerogenes or soil organisms of non-faecal origin. The complete absence of *B. coli* indicates that there were no pockets of human or animal pollution in the soil of the experimental area. The bacteriological picture is very similar to that of the ground water in its natural state (Dyer *et al.*, 1945) and may be considered satisfactory from the point of view of tracing pollution in the experimental area.

SUMMARY.

The mechanical, chemical, and bacteriological properties of the soil medium, in which ground-water pollution studies were carried out in West Bengal, India, are described.

The soil was a sandy loam typical of alluvial deposits. The surface soil contained about 20 per cent clay, which gradually decreased with increasing depths. Below 16 feet, the soil was all sand. In the water-bearing region below 14 feet this sand was a homogeneous medium with about 40 per cent pore space and properties similar to a slow sand-filter bed.

The soil was slightly acidic and well buffered by the presence of bicarbonates. It contained little organic matter and that present was of vegetable origin. Soluble minerals were not appreciable and were accounted for by carbonates, chlorides, and nitrates. Samples below 14 feet contained large amounts of free ammonia.

Bacteriological studies showed that gas-formers were present in large numbers in the soil but were definitely of non-faecal origin.

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INVESTIGATIONS OF GROUND-WATER POLLUTION.*

Part III.

GROUND-WATER POLLUTION IN WEST BENGAL, INDIA

BY

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OBJECTIVES OF THE STUDY.

THE necessity for a cheap, safe method of sewage disposal applicable to small towns and rural areas has become so pressing that investigators have been devoting much time to the problem in recent years. In India, the situation is especially acute and public health workers have repeatedly brought the question to the attention of the public (Dyer and Bhaskaran, 1943).

The results of previous investigations have not so far provided satisfactory criteria which may be used by medical workers under all varieties of conditions in the field. Soil, ground-water and latrine characteristics are primary factors determining the extent of pollution flow and they should be studied in detail in order that data may be secured for establishing the desired criteria.

Any attempt to trace pollution from a known source in the ground water must be preceded by careful studies of the character of the water, chemical and bacteriological, its velocity and direction of flow before pollution is added. A properly designed field for observations must, therefore, be laid out and a statistical plan devised for sampling and analysing the water over a period of time both before and after pollution is added. Only by this procedure may satisfactory indices of pollution be deduced for use as criteria for testing public water-supplies in the future.

Previous studies carried out in the Punjab (Dyer, 1939) showed that the ground water at shallow depths in its natural state may not be free from pollution. It was found also that degree as well as extent of such pollution had to be investigated and that the gas-forming organisms needed further differentiation.

The changes occurring in a bore-hole latrine are little understood. The extent to which excreta are disintegrated and the nature and concentration of their products have a direct bearing on the effluent. The reasons for the defence principle and for the filling up of the latrine which leads to its limited life are also obscure. No attempt has been made so far to study the relation of these events to the flow of pollution.

Although bacterial pollution is most important from the hygienic point of view the flow of chemical products of a soluble and colloidal nature are of scientific interest and must be understood if we are to have a complete picture of what is happening *in situ* within the latrine and in the ground surrounding it.

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of The Rockefeller Foundation.

In Caldwell's studies (Caldwell and Parr, 1937) pH and odour were useful indicators of chemical pollution but these constituents were little affected in the earlier studies carried out in India. Indicators which are independent of the character of the soil and sensitive to small amounts of pollution under field conditions and which are applicable to water flowing through different types of soil are needed. A more detailed study of the flow of chemical products is, therefore, required.

Investigations hitherto carried out have dealt mainly with pollution under natural conditions of ground-water flow. Circumstances may differ in actual practice. When a well in constant use is situated near a latrine, lowering the water in the well forms a cone of depression in the water-table. To re-establish its level water flows in from the surrounding area. The distance affected depends upon the soil and the draw-down caused by the pumping. This phenomenon has long been recognized and is known as the circle of influence. Pollution which might not normally reach a well may do so if it is in continuous use.

The objectives of this investigation may be summarized as follows:—

1. To make a detailed study of soil and water conditions in their natural state prior to the seeding of the latrine.
2. To investigate the direction of flow and the distance at which various constituents of pollution may be traced in the ground water following the seeding of the latrine.
3. To study the life-history of the bore-hole latrine and to determine its practicability in rural communities in India.
4. To determine the practicability of shallow wells as a source of rural water-supply.

THE SITE AND DESIGN OF THE EXPERIMENTAL FIELD.

The soil encountered in the previous studies in the Punjab (Dyer, *loc. cit.*) was highly alkaline, and it was decided that further investigations should be carried out in a neutral or slightly acid soil. The site selected was Singur, a village in West Bengal, 20 miles from Calcutta, where the soil had a hydrogen-ion concentration (pH) of from 6.3 to 6.8. It was composed of clayey silt to a depth of about 16 feet typical of the plains of India and of the Indo-Gangetic alluvium constituting them. The water-bearing sand occurred at shallow depths of 16 feet and was free from chalk.

Before it was utilized as an experimental field the plot was used mainly for cultivation of jute and potatoes. Readings of surface contours showed the site to be quite level but 75 feet below the latrine the land had been terraced for cultivation and was 3 feet lower than the rest of the area. Observation well C₃ was situated on this lower plot.

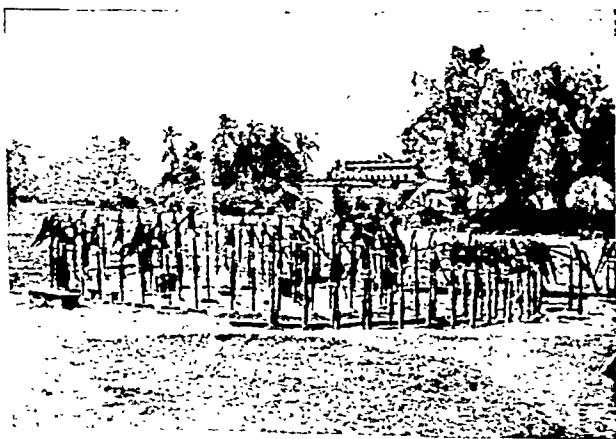
The lay-out of the field with the location of the central latrine and the encircling wells at distances of 5 and 10 feet was described in a previous paper (Dyer and Bhaskaran, 1943a). Following the determination of the direction of flow and the velocity of the ground-water rows of wells were added above and below the latrine in the direction of flow. Shallow, medium and deep wells were placed 1½ feet apart 15 feet from the latrine, medium and deep wells were placed 25 and 35 feet away and deep wells at distances of 50 and 65 feet. The design of the field is schematically indicated in Fig. 1. A general view showing the forest of wells and the type of concrete platform used to prevent surface seepage is presented in the photographs in Plate III (*see opposite*).

The notation describing the wells on the figure and in subsequent tables requires explanation. The depth of the well is indicated as S, M or D (shallow, medium or deep). In the 5-foot zone letters were used to designate the alternate shallow and medium wells. In the 10-foot zone all wells were deep and numbered consecutively. In the outer rows wells were numbered from the one on the centre line (C) drawn through the latrine the length of the field in the direction of flow and designated R (right) or L (left) of one standing at the well and facing the latrine. Thus, the second shallow well right of centre in the 15-foot zone was designated SR₂, the medium well left in the corresponding position ML₂.

Wells 25 and 50 feet above the latrine counter to the direction of flow have been given the prefix X.

PLATE III.

The location and arrangement of latrine and wells in the 5-, 10- and 15-foot zones at the experimental site, Singur, West Bengal, India.



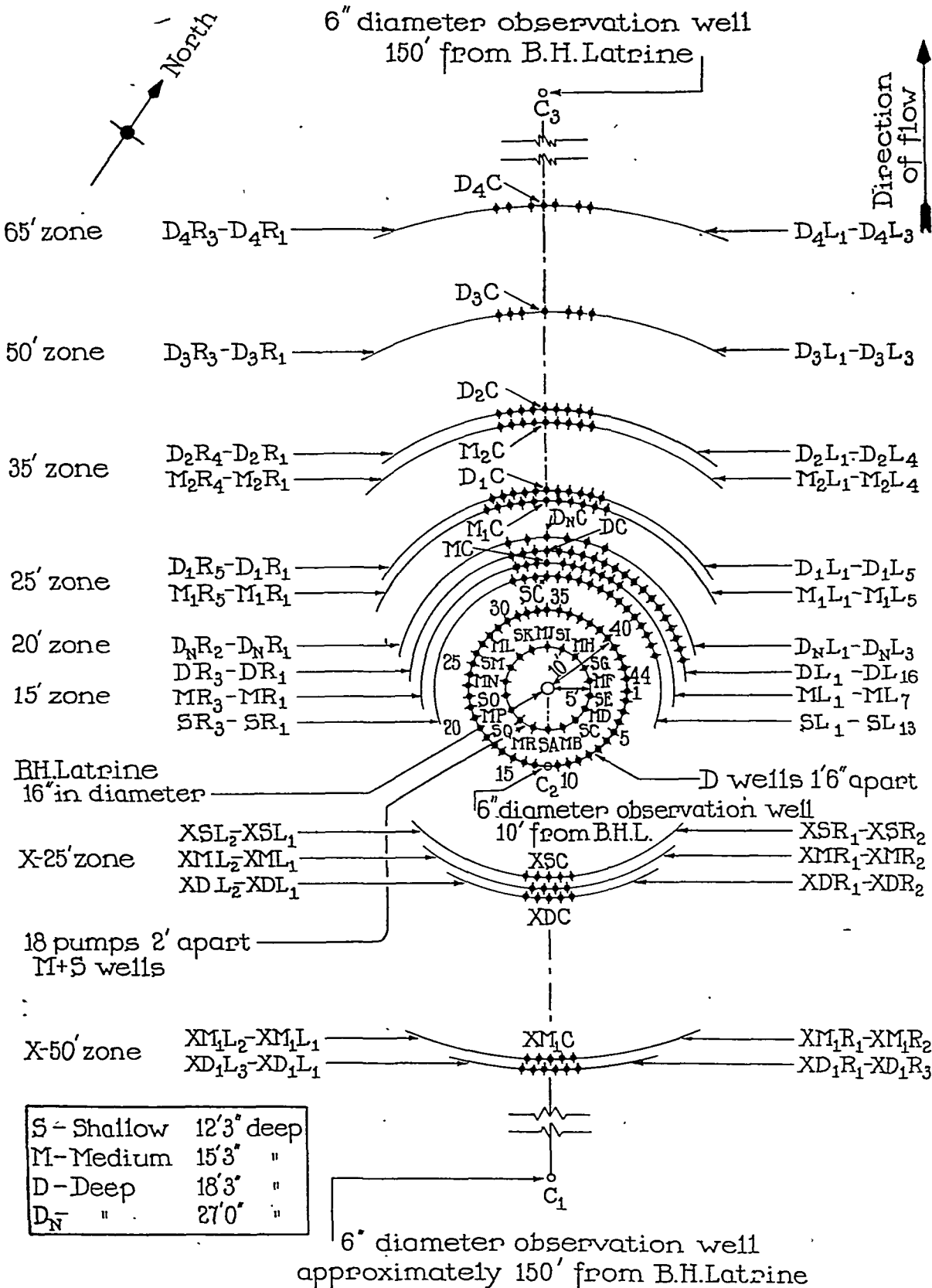
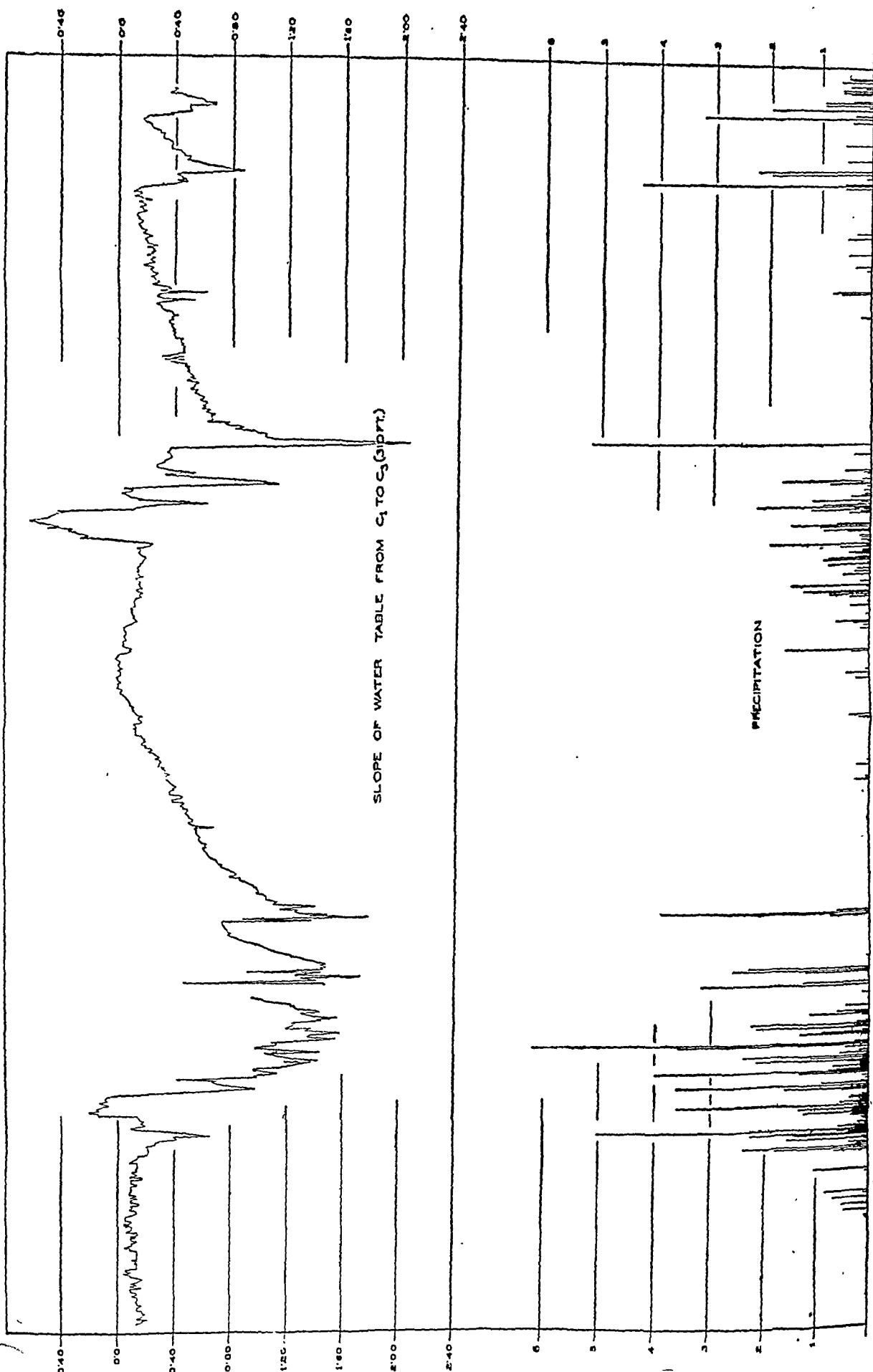


FIG. 1.

Difference in water level ($C_1 - C_3$) in f

Rainfall in inches.



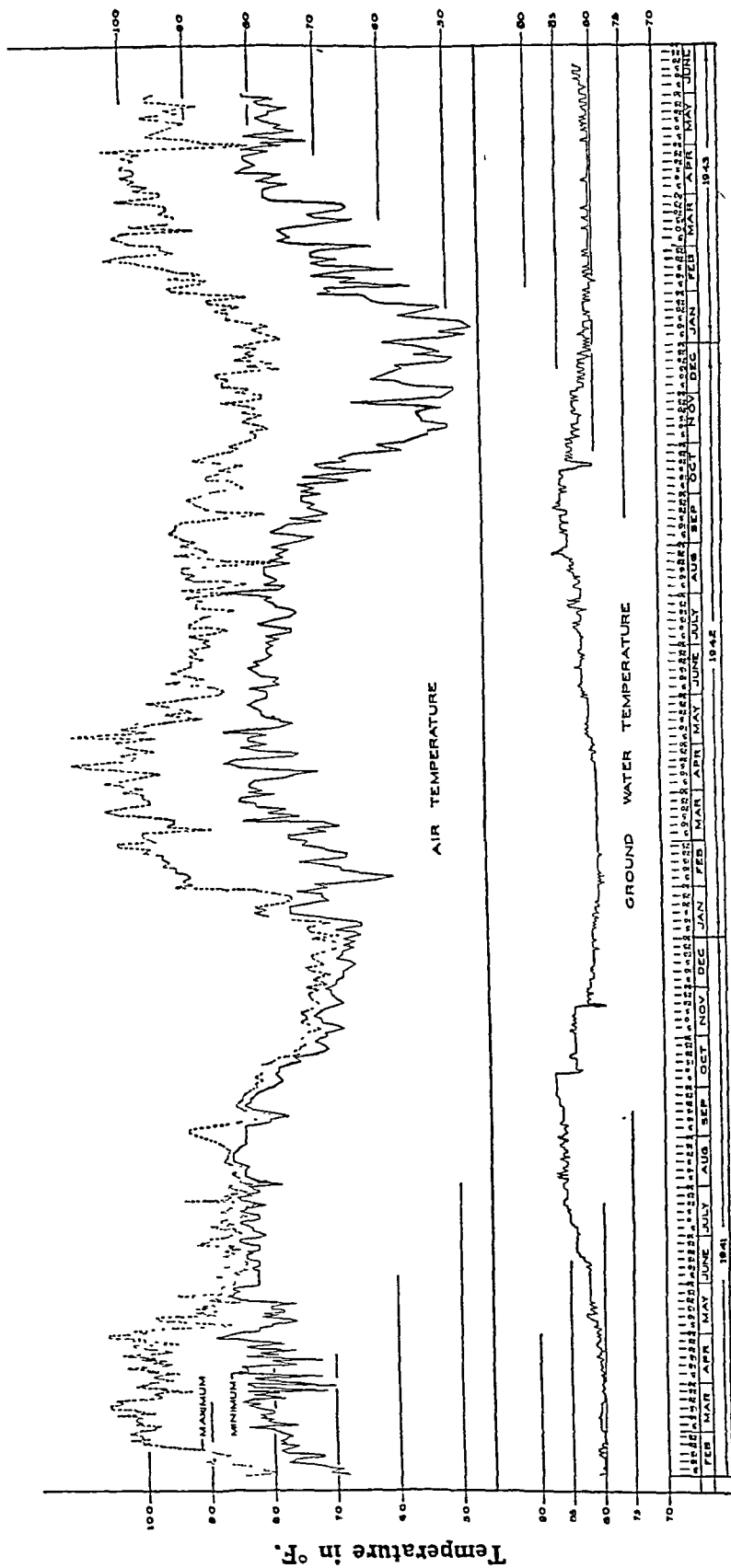


Fig. 2.--Ground-water and meteorological conditions.

RESULTS.

Character of the soil.—The results of the soil analyses have been discussed elsewhere (Dyer and Bhaskaran, 1943a; 1945). Soil at the surface contained about 20 per cent clay which gradually decreased in amount with increasing depths. Below 16 feet the soil was all sand. In the water bearing region below 14 feet the soil was a homogeneous medium with about 40 per cent pore space and properties similar to a slow sand-filter bed. Although there were significant differences between strata, the soil structure for the site as a whole was homogeneous.

Direction of flow and velocity.—The salt added to the central bore-hole latrine (Dyer and Bhaskaran, 1943a) was recovered in the 5-foot zone in shallow wells SM, SK, SI and SG and in medium wells ML, MJ and MH (see Fig. 1). Concentration was higher in wells SK, SI and ML. In the 10-foot zone the salt content of the water rose in wells D₂₇ to D₄₀ but was more concentrated in wells D₃₃ and D₃₁. Judging from the rapidity with which salt appeared in the medium and deep wells the velocity of flow might have been as high as 2½ feet per day. The velocity in the S stratum was somewhat less.

Ground-water and meteorological conditions.—The water-table and its slope influence the flow of pollution and are themselves related to rainfall, air and water temperature and humidity. Systematic observations were made throughout the period of the experiment and are shown in Fig. 2.

The days were generally quite humid. Daily readings taken at 8 a.m. varied from 50 to 100 per cent with humidity over 80 per cent during most of the period. The high humidity and the presence of clayey soil on the surface which was fully saturated may have decreased the percolation of rain into the ground water.

There was little variation in ground-water temperature when compared with atmospheric temperatures. Seasonal changes may not, therefore, have had a pronounced effect on the decomposition of biological constituents of the ground water and the latrine.

Rainfall varied from 0.98 inches in January 1941 to 22.09 inches in June, 25.04 inches in August and 6.14 in October. In January 1942, there was no rain, in June, 1.78 and in August 10.25 inches. In January 1943, there was 1.67 and in June, 12.37 inches of rain. In spite of the variations in the amount of rainfall, fluctuations in the height of the ground-water table were similar in the different years. This is shown by the readings in observation well C₁ in Fig. 3:—

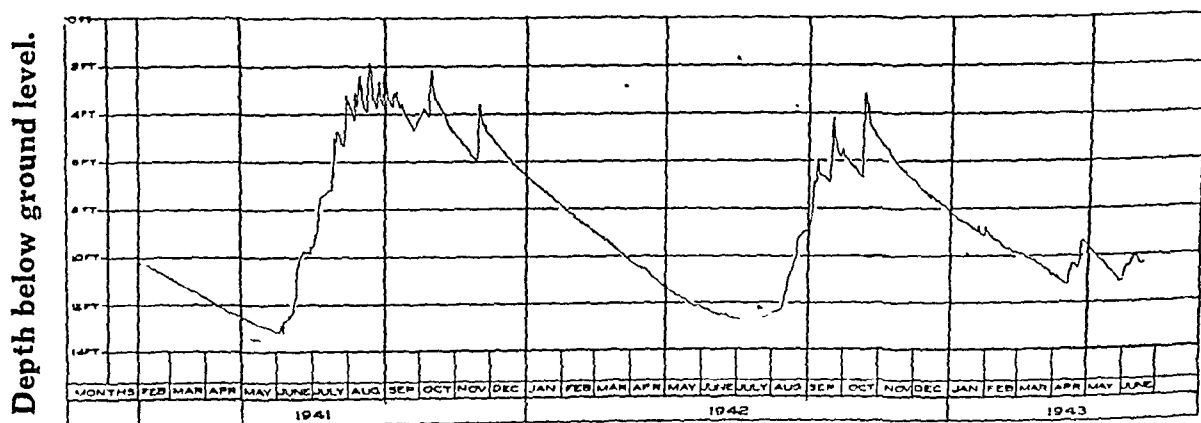


FIG. 3.—Water-table in observation well C₁.

Fluctuations in slope were also similar in the different years and are shown in Fig. 2. The maximum slope each year was of the order of 1/200. During the dry months the water-table became very flat.

True and false water-table.—When borings were made in the soil it was observed that water could not be struck at levels recorded for the observation wells. The true ground-water table was always lower. The importance of large differences between true and false water levels in studies on pollution flow from the latrine is obvious. Therefore, made near observation wells C₁, C₂ and C₃ and the

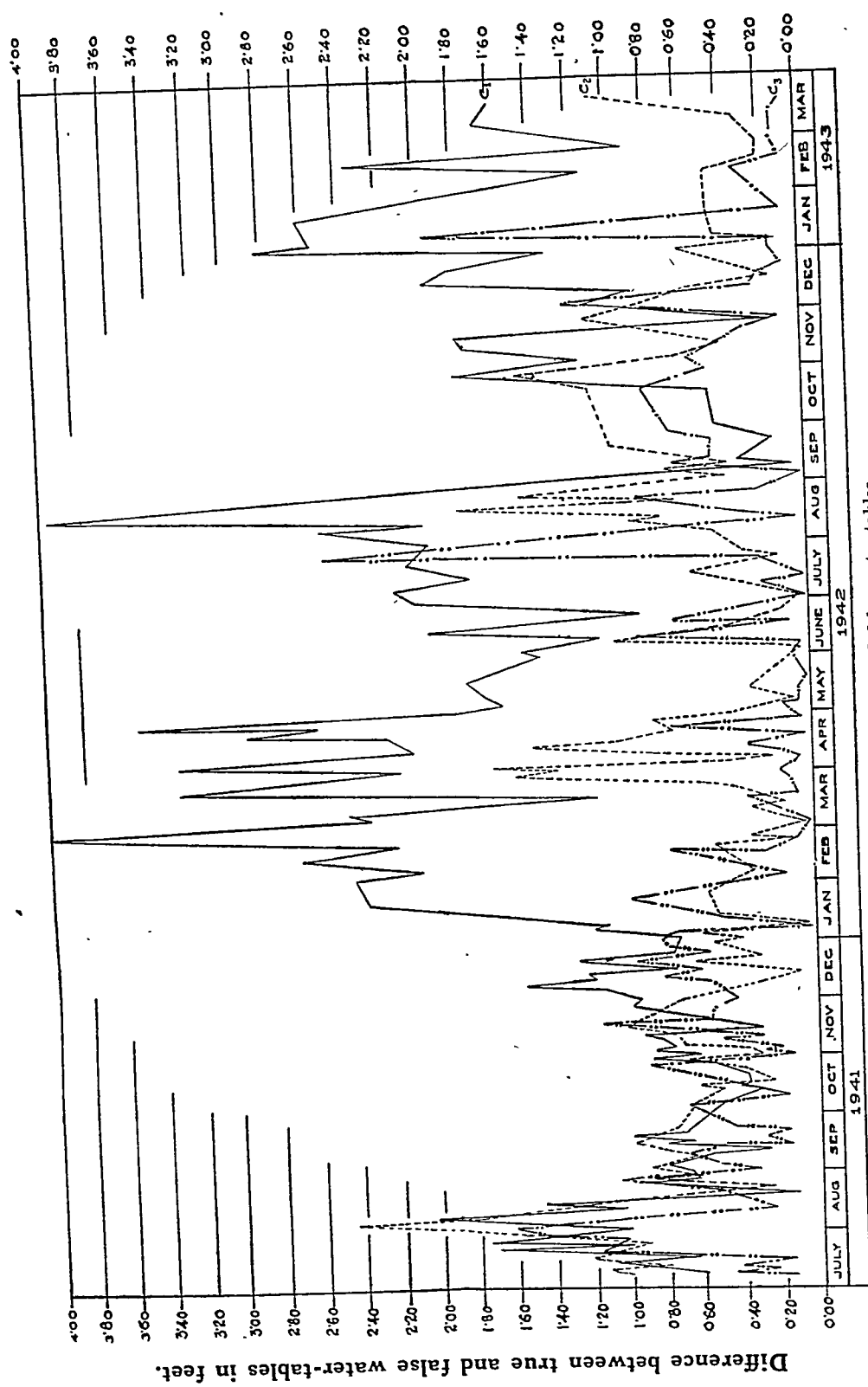


Fig. 4.—True and false water-tables.

The results show that differences in the area around well C₂ were not great enough to affect the flow from the latrine. The nature of the soil in which the water-table stood accounted for the observed differences. Near wells C₁ and C₂ where the water-table occupied clayey strata differences were greater than near well C₃ where it lay in a sandy medium.

Latrine conditions after seeding.—In their Alabama studies Caldwell and Parr (*loc. cit.*) observed that in spite of the daily seeding, the latrine contents and the surrounding ground water stood at the same level. The use of a .2-inch observation well only one foot from the latrine to record the height of the water-table may have accounted for this fact.

In the Singur study the latrine contents rose rapidly due to their concentration and to the formation of a colloidal barrier analogous to the defense mechanism. The seeding procedure was, therefore, altered on the 3rd March, 1942, after which the night-soil was diluted with an equal amount of water before it was dumped into the latrine. In spite of this dilution it was found necessary to stop seeding for a few days to prevent the latrine from overflowing. After the 20th July, 1942, seeding was discontinued as soon as the water surface rose to 2-feet below the surface of the ground and 4 gallons of water were added daily until the level dropped to 4 feet, when seeding was resumed. Actually the latrine was seeded only about 330 days during the 1½ years of the study. Days when seeding occurred are indicated in Fig. 5.

Two days after seeding began the latrine was observed to be overgrown with chrysonia larvæ. They were originally present in the night-soil (common in latrines of these localities) and multiplied abundantly in the latrine. They continued to be present throughout the period of the investigation although the concentration was less during certain parts of the year.

There was little septic action during the first few months but from March 1942 onward vigorous septic action was observed with profuse gas-production, and a distinct mat gradually formed on the surface. During the hot months of March, April and May 1942, the scum-mat thickened to about a foot. Below this the contents were mushy consisting of blackish digested faecal material. The sludge at the bottom was still rather loosely packed and could not be distinguished from the layer above. Colder months had little effect on the septic process which continued with the same vigour so that the latrine looked like a well-controlled septic tank. By November 1942, there was a 2-foot column of packed sludge at the bottom.

Latrine contents were sampled at two depths and the results of chemical and bacteriological tests are presented in Tables I and II.

The chemical findings indicate that the pH of the medium became alkaline in April and thereafter continued to be 7.5 to 8.0, favourable for good septic action. The soluble minerals as shown by conductivity increased during March-June 1942, and later decreased. Alkalinity and the ammonias showed the same trend. Nitrates and chlorides showed little increase. Bacteriological findings show that the gas-formers which were present in large numbers throughout the period were mostly *B. coli*. Organisms were distributed throughout the latrine although the concentration was greater at the bottom than in the middle layers. *C. welchii* was also detected in large numbers.

The chemical findings support the observations on physical status in the bore-hole latrine. With the onset of septic action in March-April 1942, more minerals and ammonia were released. High free ammonia in May and June showed good ammonification. Later, with the outflow of pollution it decreased in amount as did also that of the other soluble constituents. The stationary nitrate content indicates that there was little nitrification in the latrine and that the septic process proceeded only to the ammonia stage.

Apparently only a fraction of the excreta added to the latrine disintegrated. At the end of the experiment the accumulated sludge and solids were estimated at 5.5 cubic feet. This would occupy a 7-foot column in a 12-inch bore-hole.

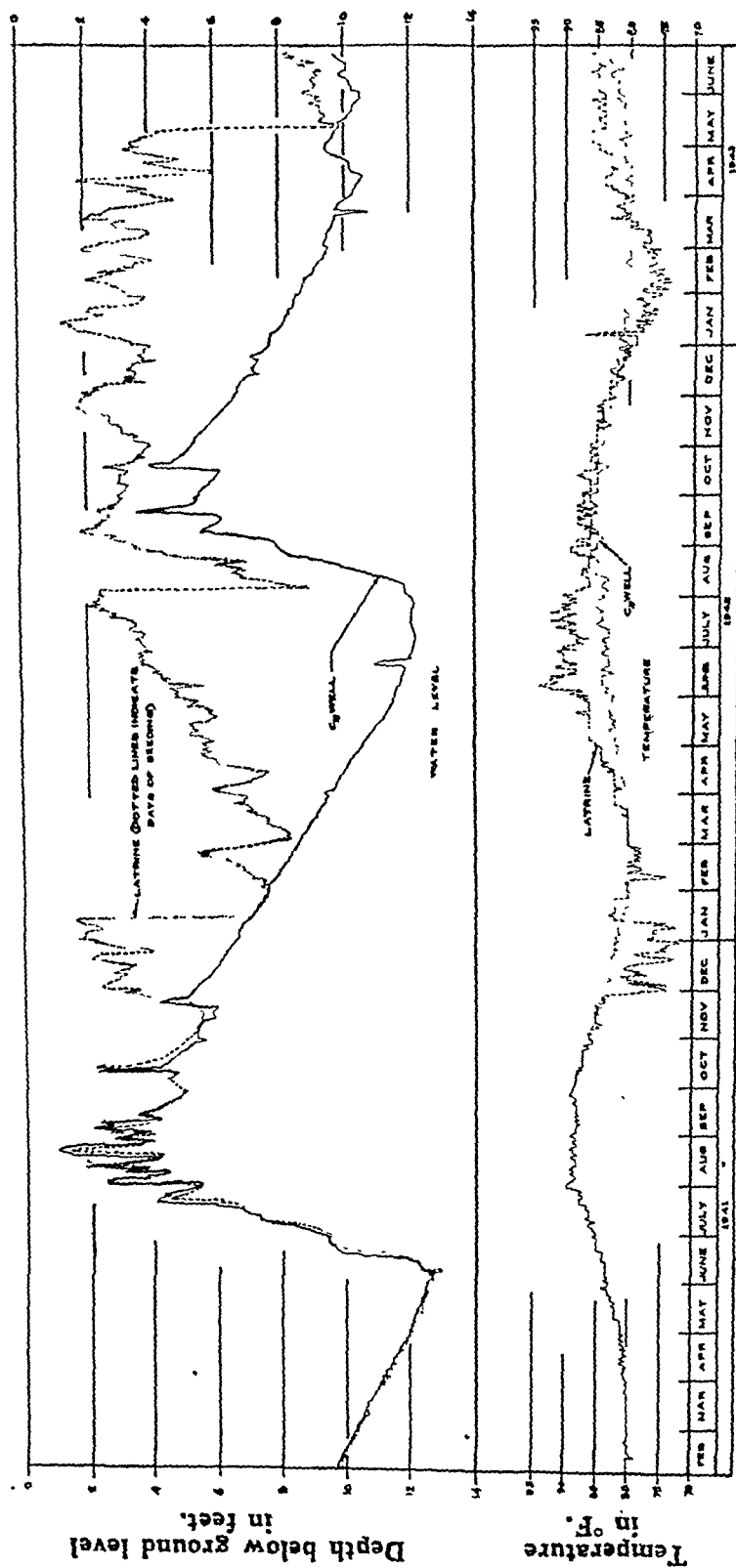


FIG. 5.—Water-table and temperature in latrine and observation well C.

TABLE I.

Chemical analyses of the water-soluble constituents in the latrine after seeping.

Period.	Number of observations.	pH.	MEAN VALUE OF SAMPLES COLLECTED AT TWO DEPTHS (16 FT. AND BOTTOM OF LATRINE).							
			Conductivity ($1/10^6$ ohms).	Chlorides (ppht) NaCl.	Alkalinity (ppm CaCO_3).	Acidity (ppm CaCO_3).	Nitrites (ppm NO_2).	Nitrates (ppm NO_3).	Free ammonia (ppm NH_3).	Albuminoid ammonia (ppm NH_3).
33 Jan.-Feb. 1942	3	6.3	12,625	130.4	4,933	1,200	7.50	166.8	912	771.0
34 March-April 1942	7	7.4	10,732	126.5	4,558	879	1.43	104.5	747	525.0
May-June 1942	12	8.0	17,200	126.5	7,250	680	0.00	85.0	1,215	567.5
July-Aug. 1942	12	8.0	11,300	85.3	4,980	640	0.33	60.3	1,070	673.0
Sep.-Oct. 1942	12	8.0	6,437	38.3	3,000	240	1.50	13.9	661	146.3
Nov.-Dec. 1942	8	7.4	5,118	22.3	2,590	415	0.00	25.3	497	50.0
Jan.-Feb. 1943	6	7.7	5,825	37.7	2,700	520	0.00	20.5	512	42.0
March-April 1943	10	7.7	4,720	51.0	1,800	340	0.00	14.2	376	91.0

TABLE II.

Bacteriological analysis of latrine contents after seeding.

Period.	Number of observations.	M.P.N. (GAS-FORMERS PER THOUSAND PER C.C.).		NATURE OF GAS-FORMERS.	
		16-foot depth.	Bottom.	16-foot depth.	Bottom.
Nov.-Dec. 1941 ..	7	280	327	<i>B. coli.</i>	<i>B. coli</i> , intermediate II.
Jan.-Feb. 1942 ..	3	190	257	<i>B. coli</i> , <i>aerogenes</i> II.	<i>B. coli</i> , <i>aerogenes</i> II.
March-April 1942 ..	7	630	521	<i>B. coli.</i>	<i>B. coli.</i>
May-June 1942 ..	9	139	216	<i>B. coli</i> , intermediate II.	<i>B. coli</i> , intermediate II, irregular & soil organisms.
July-Aug. 1942 ..	7	42	65	<i>B. coli</i> , <i>aerogenes</i> II and other soil organisms.	<i>B. coli.</i>
Sep.-Oct. 1942 ..	6	5	6	<i>B. coli</i> , <i>aerogenes</i> I and intermediate II.	<i>B. coli</i> , intermediate II and irregular &.
Nov.-Dec. 1942 ..	4	20	39	<i>B. coli</i> , intermediate II.	<i>B. coli</i> , intermediate II.
Jan.-Feb. 1943 ..	3	29	19	<i>B. coli.</i>	<i>B. coli</i> , intermediate II and soil organisms.
March-April 1943 ..	5	54	39	<i>B. coli</i> , <i>aerogenes</i> .	<i>B. coli</i> , <i>aerogenes</i> I.

Indices of chemical pollution.—Experimental errors involved in routine chemical analyses of water samples must be considered in judging the relative value of the different tests for chemical pollution. A number of factorial analyses were, therefore, carried out to estimate the error due to variation of (1) different samples from the same draw, (2) different samples from different draws, (3) same as (2) with longer intervals between draws, (4) personal factor and (5) storage of samples for short periods extending up to 6 hours. Conductivity and acidity figures only were tested for these errors as these appeared to be more sensitive in detecting chemical pollution. The results are summarized in Table III:—

TABLE III.

Magnitude of standard deviation and coefficient of variation.

Source of error (see text).	STANDARD DEVIATION.		COEFFICIENT OF VARIATION (PER CENT).	
	Conductivity.	Acidity.	Conductivity.	Acidity.
(1)	2.24	6.07	0.8	8.0
(1) and (2) ..	4.73	7.45	1.6	9.8
(1), (2) and (3) ..	4.87	7.46	1.6	9.8
(4)	2.82	5.01	1.0	6.6
(1), (2), (3) and (4) ..	5.63	8.98	1.9	11.8
(1) and (5) ..	25.02	26.20	8.5	34.5

Table III shows that excluding the error due to storage (5) the coefficient of variation for conductivity was very small. Even with this factor added, an event which rarely happened; conductivity consistently varied less than acidity.

A good test should be characterized not only by a small experimental error but also by an immediate response to small amounts of pollution. Since it is a measure of all soluble salts, conductivity changes due to pollution are likely to be larger than those given by other chemical tests. Conductivity may, therefore, prove to be a more useful test for tracing chemical pollution.

Chemical pollution prior to seeding.—The results of tests for pH, conductivity, acidity and the ammonias made on well samples in the preliminary survey were analysed statistically to determine the relative importance of the three factors, depth of well, zone and period (round). Results of the variance analysis and the mean values based on samples from the five replicate wells are given in Table IV.

The variability of test results from sample to sample as shown by the standard deviations was greatest for conductivity with that for acidity next. Depth of well and round were factors significant at the 1 per cent level for each constituent except pH. Differences due to zones were of a lower order but were significant except those for tests for albuminoid ammonia.

Wells in the 5- and 10-foot zones contained comparatively larger and more varying amounts of chlorides because of the salt added to the latrine in the velocity experiments (Dyer and Bhaskaran, 1943a) and these figures were not analysed. The chloride content of the majority of wells was less than 2.5 parts per hundred thousand (ppht). Nitrites were present in quantities less than 0.01 parts per million (ppm) and nitrate content varied from 0.08 to 0.12 parts per million.

Degree and extent of chemical pollution after seeding.—Five thousand water samples were collected from wells and analysed for chemical pollution after the latrine had been seeded. Mean conductivity and acidity of wells in the direction of ground-water flow for five periods (November-December, 1941; January-April, May-August, September-December, 1942; January-April, 1943) are presented in Tables V to VII. The period in which pollution was established and its intensity are indicated for each well.

It may be observed that a rise in conductivity designated more wells as polluted than did a rise in acidity and also that the conductivity increase was proportionately greater. The possible effect of salt added for the velocity experiment on conductivity was considered but when conductivity values were corrected for the salt effect the conclusions were the same. The nature of the day-to-day fluctuations in conductivity in some of the wells is presented in Fig. 6.

With conductivity values as a basis wells were grouped into those polluted and those non-polluted and the mean concentration of various chemical constituents for each of the four periods was computed. The values are shown in Tables VIII to X. Those for non-polluted wells precede those for polluted wells in each zone.

The presence of chemical pollution as indicated by the rises in conductivity was corroborated by changes in all the other constituents examined with the exception of pH and nitrates. The relative sensitivity of other indicators differed, however, especially when values were contrasted with their standard deviations. Alkalinity is apparently in no way inferior to conductivity as a test for pollution. Acidity is next in importance although the large experimental error (Table III) renders it a less useful indicator. Ammonias are least sensitive probably because of the large amounts present in the ground water in its natural state. pH was not affected by pollution because of the good buffering of the medium due to the presence of bicarbonates. There was little nitrification in the latrine and no nitrates were recovered in the effluent.

The recovery of pollution in wells as shown by the chemical tests is summarized in Table XI.

TABLE IV.

Chemical analyses of water samples and their significance, prior to seeding of the larvae.

MEANS BASED ON SAMPLES FROM 5 WELLS.														
Chemical constituents.	Zones.	SHALLOW.			MEDIUM.			DEEP.			Standard deviation of individual observation.	Coefficient of variation (per cent).	SIGNIFICANCE OF FACTORS.	
		1 (July)	2 (Aug.)	3 (Sep.)	1 (July)	2 (Aug.)	3 (Sep.)	1 (July)	2 (Aug.)	3 (Sep.)				
pH	{ Z ₁ Z ₂ Z ₃ Z ₄	{ 6.70 6.72 6.70 6.76	{ 6.68 6.66 6.74 6.72	{ 6.70 6.68 6.72 6.72	{ 6.74 6.68 6.72 6.78	{ 6.74 6.68 6.72 6.78	{ 6.74 6.70 6.72 6.80	{ 6.76 6.72 6.70 6.76	{ 6.70 6.74 6.64 6.70	{ 6.72 6.74 6.64 6.70	0.08	1.25	*	—
Conductivity (1/10° ohms).	{ Z ₁ Z ₂ Z ₃ Z ₄	{ 297 278 323 298	{ 230 260 263 263	{ 209 224 261 241	{ 293 278 273 284	{ 287 260 301 288	{ 283 236 255 201	{ 253 252 239 232	{ 252 266 245 230	{ 238 233 230 236	22.15	8.46	**	**
Acidity (ppm CaCO ₃)	{ Z ₁ Z ₂ Z ₃ Z ₄	{ 74.4 81.2 77.2 70.2	{ 78.8 77.4 79.2 60.2	{ 60.4 68.4 65.4 53.0	{ 70.2 64.4 67.4 57.6	{ 66.8 70.2 81.6 64.2	{ 66.4 57.4 51.2 63.4	{ 52.4 57.0 60.2 51.0	{ 56.4 60.8 66.2 58.4	{ 54.0 58.2 59.2 53.4	12.22	18.95	**	**
Free ammonia (ppm NH ₃).	{ Z ₁ Z ₂ Z ₃ Z ₄	{ 3.46 3.06 2.79 2.63	{ 1.97 2.17 2.60 1.89	{ 1.33 1.97 2.31 1.28	{ 3.07 3.67 2.49 2.28	{ 2.82 2.66 2.91 2.44	{ 2.72 2.64 2.17 2.50	{ 1.20 1.02 0.96 0.66	{ 1.14 1.37 0.90 0.77	{ 1.24 1.59 1.40 1.04	0.66	32.49	**	**
Albuminoid ammonia (ppm NH ₃).	{ Z ₁ Z ₂ Z ₃ Z ₄	{ 0.11 0.16 0.21 0.12	{ 0.07 0.11 0.12 0.09	{ 0.07 0.07 0.08 0.07	{ 0.08 0.15 0.13 0.11	{ 0.10 0.10 0.09 0.11	{ 0.09 0.09 0.08 0.09	{ 0.08 0.10 0.09 0.08	{ 0.07 0.06 0.04 0.09	{ 0.06 0.07 0.06 0.05	0.07	77.00	**	—

N.B. — indicates not significant at 5 per cent probability limit.

* indicates significant at 5 per cent probability limit.

** indicates significant at 5 per cent probability limit.

TABLE
Flow of chemical pollution in shallow strata

		CONDUCTIVITY (1/10 ² ohms).										
Distance in feet from latrine.	Wells.	Nov.-Dec. 1911.		Jan.-Apr. 1912.		May-Aug. 1912.		Sept.-Dec. 1912.		Jan.-Apr. 1913.		
		N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	
5	..	SA	4	203.8	4	240.0	3	260.0	4	256.2	4	230.0
		SC	7	245.0	16	239.4	18	251.1	14	292.1	10	264.0
		SE	19	249.5	15	246.0	16	292.5	14	283.6	11	258.6
		SG	13	225.0	30	314.2	22	618.2	15	512.7	11	486.8
		SI	35	249.0	43	328.1	26	519.2	16	527.8	11	558.2
		SK	17	273.5	20	298.2	18	336.9	13	370.4	11	326.8
		SM	19	246.3	19	264.7	16	282.5	5	289.0	3	283.3
		SO	3	236.7	8	267.5	3	285.0	4	272.5	4	270.0
	SQ	3	233.3	3	250.0	3	275.0	4	243.8	5	262.0	
15	..	SR ₁	3	271.7	2	280.0	1	300.0	13	342.3	11	309.5
		SR ₂	2	262.5	1	375.0	2	345.0	12	340.4	10	349.0
		SR ₁	3	248.3	1	250.0	14	368.6	12	355.4	11	381.8
		StC	1	250.0	1	280.0	12	287.1	14	327.5	11	376.8
		SL ₁	1	260.0	13	279.2	13	313.5	11	350.5
		SL ₂	1	280.0	11	297.3	12	316.2	10	328.5
		SL ₃	6	251.7	2	255.0	13	248.5	12	293.8	11	303.2
		SL ₄	11	256.4	13	298.5	11	301.8
		SL ₅	3	300.0	15	308.7	11	300.0	11	342.3
		SL ₆	2	240.0	10	253.0	13	288.5	9	302.8
		SL ₇	3	268.3	12	270.8	11	293.6	11	319.1
		SL ₈	7	306.4	13	303.8	11	307.3
		SL ₉	8	270.6	13	280.8	11	281.8
		SL ₁₀	6	241.7	13	243.1	10	226.0
		SL ₁₁	6	285.0	13	279.6	11	289.5
SL ₁₂	7	275.7	13	244.6	11	245.0		
	SL ₁₃	6	265.8	11	232.3	11	229.1	

N indicates number
Italicized figures indicate
Antique figures indicate

V.

*after seeding the latrine.*ACIDITY (ppm CaCO_3).

NOV.-DEC. 1941.		JAN.-APR. 1942.		MAY-AUG. 1942.		SEPT.-DEC. 1942.		JAN.-APR. 1943.	
N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
4	66.0	4	54.0	3	64.7	4	56.0	4	58.0
7	68.9	16	55.5	18	57.7	14	84.0	10	60.0
19	68.1	15	44.3	16	51.1	14	64.0	11	49.5
13	76.6	30	74.3	22	151.5	15	115.5	11	108.7
35	63.0	43	60.6	26	97.5	16	114.8	11	125.5
17	68.9	20	52.5	18	57.1	13	72.3	11	57.8
19	66.1	19	54.5	16	53.4	5	51.2	3	46.7
3	60.0	8	56.0	3	54.0	4	51.0	4	53.0
3	64.7	3	68.7	3	62.0	4	56.0	5	67.2
3	58.7	2	58.0	1	50.0	13	64.3	11	53.8
2	43.0	1	52.0	2	48.0	12	56.8	10	58.8
3	56.7	1	46.0	14	57.0	12	61.0	11	62.9
1	77.0	1	50.0	12	50.2	14	67.1	11	73.8
..	..	1	32.0	13	44.3	13	57.8	11	61.1
..	..	1	64.0	11	52.9	12	60.7	10	62.0
6	66.2	2	50.0	13	42.8	12	62.5	11	51.6
..	11	32.5	13	43.8	11	52.4
..	..	3	38.0	15	40.5	11	52.5	11	50.5
..	..	2	43.0	10	42.2	13	51.2	9	54.7
..	..	3	57.3	12	49.7	11	65.3	11	58.4
..	7	64.6	13	58.0	11	70.5
..	8	52.5	13	51.4	11	55.6
..	6	41.3	13	48.6	10	55.2
..	6	52.0	12	59.5	11	55.3
..	7	56.9	13	60.9	11	73.5
..	6	56.7	11	59.8	11	47.3

of observations.
evidence of pollution.
evidence of intense pollution.

*Flow of chemical pollution in medium strata*CONDUCTIVITY (1/10³ ohms).

Distance in feet from latrine.	Wells.	Nov.-Dec. 1941.		Jan.-Apr. 1942.		May-Aug. 1942.		Sept.-Dec. 1942.		Jan.-Apr. 1943.	
		N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
5	MB	2	195.0	4	232.5	3	206.7	4	243.8	4	228.8
	MD	1	275.0	2	257.5	18	266.3	13	271.2	11	260.9
	ME	17	290.1	15	300.3	15	283.8	14	402.9	10	284.0
	MH	17	283.5	32	397.7	22	425.9	16	388.4	11	348.2
	MJ	35	271.9	31	295.8	24	301.7	15	368.7	10	286.5
	ML	16	234.7	17	279.7	17	283.5	14	297.5	11	267.7
	MN	19	260.8	20	300.5	6	300.0	5	291.0	4	295.0
	MP	4	270.0	5	296.0	3	286.7	4	297.5	4	292.5
	MR	2	255.0	2	280.0	4	303.8	3	280.0	4	276.2
15	MR ₃	4	281.2	3	300.0	2	312.5	12	312.9	9	303.3
	MR ₂	2	275.0	2	305.0	2	300.0	13	303.5	12	302.9
	MR ₁	3	263.3	2	290.0	12	298.8	12	303.8	11	275.9
	MC	1	25.50	1	275.0	12	298.3	13	<i>314.2</i>	10	294.0
	ML ₁	12	315.8	12	<i>338.8</i>	11	308.6
	ML ₂	1	275.0	10	283.0	13	296.2	10	248.0
	ML ₃	2	275.0	1	290.0	12	303.8	12	<i>323.8</i>	13	284.6
	ML ₄	3	303.3	12	315.8	13	<i>338.1</i>	11	305.9
	ML ₅	1	300.0	11	308.2	13	312.3	11	327.3
	ML ₆	2	300.0	12	312.1	13	321.9	11	367.3
	ML ₇	2	290.0	11	288.6	13	306.9	11	296.4

N indicates number
Italicized figures indicate
Antique figures indicate

VI.

after seeding the latrine.

ACIDITY (ppm CaCO_3).

Nov.-Dec. 1941.		Jan.-Apr. 1942.		May-Aug. 1942.		Sept.-Dec. 1942.		Jan.-Apr. 1943.	
N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
2	53.0	4	40.0	3	37.3	4	40.0	4	51.0
1	66.0	2	45.0	18	42.4	13	60.0	11	53.1
17	79.8	15	63.1	15	56.9	14	91.7	10	54.4
17	82.9	32	83.2	22	93.9	16	95.6	11	73.1
35	75.5	31	69.0	24	71.2	15	87.3	10	69.6
16	66.1	17	62.1	17	59.4	14	54.9	11	56.7
10	73.9	20	64.8	6	51.3	5	62.8	4	52.0
4	65.0	5	48.8	3	60.0	4	62.5	4	48.0
2	72.0	2	53.0	4	69.5	3	56.0	4	64.0
4	53.0	3	38.7	2	43.0	12	55.5	9	42.7
2	41.0	2	31.0	2	34.0	13	49.5	12	47.0
3	70.0	2	60.0	12	63.5	12	65.3	11	56.7
1	77.0	1	42.0	12	50.5	13	51.7	10	54.8
..	12	52.3	12	69.2	11	54.2
..	..	1	38.0	10	46.0	13	49.7	10	42.8
2	64.0	1	58.0	12	60.5	12	63.5	13	54.2
..	..	3	50.7	12	48.5	13	52.9	11	57.5
..	..	1	40.0	11	40.9	13	49.5	11	60.7
..	..	2	47.0	12	61.8	13	60.9	11	76.7
..	..	2	45.0	11	39.3	13	53.1	11	49.8

of observations.
evidence of pollution.
evidence of intense pollution.

TABLE
Flow of chemical pollution in deep

		CONDUCTIVITY (1/10° ohms).									
		Nov.-DEC. 1941. JAN.-APR. 1942. MAY-AUG. 1942. SEP.-DEC. 1942. JAN.-APR. 1943.									
Distance in feet from latrine.	Wells.	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
..	D ₃₃	17	231.2	18	222.5	10	230.0	14	230.0	10	234.0
	D ₃₄	18	228.3	18	224.2	16	221.2	14	232.9	11	223.6
	D ₃₅	17	244.1	19	237.0	16	240.6	13	251.9	11	243.6
	D ₃₆	18	245.0	19	243.2	15	263.3	15	273.0	11	238.8
	D ₃₇	17	240.6	21	244.3	9	240.0	14	310.4	10	346.0
	D ₃₈	18	249.7	20	246.5	19	244.7	13	301.2	11	260.0
	D ₃₉	16	238.8	21	237.1	15	245.0	14	271.8	11	254.1
	D ₄₀	11	254.5	16	315.3	14	284.6	11	248.2
	D ₄₁	10	233.5	15	273.3	14	293.2	10	252.5
	D ₄₂	12	222.1	16	225.2	13	284.6	11	249.5
	D ₄₃	10	229.5	16	229.1	13	281.5	11	248.2
	D ₄₄	4	225.0	10	235.0	14	287.9	11	253.6
	D ₁	1	260.0	1	250.0	13	299.5	10	267.5
	D ₂	4	263.8	6	258.3	1	250.0	13	313.5	10	278.0
	D ₃	2	245.0	2	237.5	12	304.2	10	252.5
	D ₄	1	250.0	2	300.0	12	292.9	11	255.5
..	D ₅	1	300.0	14	328.6	11	264.5
	D ₆	2	227.5	12	264.2	11	254.1
	D ₇	2	250.0	1	250.0	13	278.1	10	275.5
	DL ₂	1	260.0	2	230.0	13	230.0	3	246.7	5	226.0
	DL ₃	2	235.0	10	235.0	3	251.7	4	232.5
	DL ₄	1	240.0	13	244.2	3	270.0	4	247.5
	DL ₅	2	222.5	12	220.4	2	330.0	4	241.2
	DL ₆	1	250.0	13	251.2	4	317.5	4	287.5
..	DL ₇	2	215.0	13	216.2	3	286.7	4	280.0
	DL ₈	5	258.0	3	276.7	4	281.2
	DL ₉	6	255.0	6	296.7	4	263.8
	DL ₁₀	7	232.9	4	267.5	4	250.0
	DL ₁₁	6	263.3	4	293.8	4	275.0
	DL ₁₂	7	238.6	4	283.8	3	268.3
	DL ₁₃	6	226.7	4	290.0	3	246.7
	DL ₁₄	5	237.0	4	258.8	4	237.5
	DL ₁₅	7	238.6	4	275.0	4	263.8
	DL ₁₆	6	228.3	4	266.2	3	270.0

N indicates number
Italicized figures indicate
Antique figures indicate

VII.

strata after seeding the latrine.

ACIDITY (ppm CaCO ₃).									
NOV.-DEC. 1941.		JAN.-APR. 1942.		MAY-AUG. 1942.		SEP.-DEC. 1942.		JAN.-APR. 1943.	
N	Mean	N	Mean.	N	Mean	N	Mean	N	Mean
17	65.2	18	50.1	10	54.2	14	52.0	10	56.8
18	67.0	18	51.4	16	48.6	14	47.9	11	50.9
17	69.4	19	58.1	16	56.1	13	65.4	11	60.7
18	79.6	19	57.3	15	58.7	15	74.5	11	53.5
17	76.2	21	61.4	9	53.6	14	79.3	10	79.6
18	77.2	20	57.1	18	54.8	13	70.6	11	69.5
16	66.9	21	50.5	15	49.7	14	66.4	11	60.0
..	..	11	48.0	16	61.2	14	65.4	11	51.6
..	..	10	56.4	15	61.1	14	85.7	10	67.6
..	..	12	47.5	16	47.2	13	70.0	11	60.0
..	..	10	55.6	16	53.6	13	63.2	11	62.5
..	..	4	46.5	10	49.4	14	69.9	11	60.4
1	74.0	1	40.0	13	66.9	10	61.2
4	84.5	6	64.3	1	60.0	13	81.2	10	66.8
..	..	2	49.0	2	49.0	12	66.3	10	65.2
..	..	1	58.0	2	65.0	12	77.3	11	62.9
..	1	32.0	14	55.9	11	48.0
..	2	35.0	12	54.7	11	56.4
..	..	2	33.0	1	40.0	13	52.6	10	52.8
1	60.0	2	46.0	13	49.1	3	45.3	5	57.6
2	56.0	10	50.4	3	54.0	4	61.0
..	..	1	34.0	13	38.5	3	60.0	4	47.0
..	..	2	24.0	12	28.5	2	63.0	4	37.0
..	..	1	40.0	13	51.2	4	72.0	4	60.0
..	..	2	25.0	13	27.1	3	57.3	4	48.0
..	5	46.8	3	43.3	4	64.0
..	6	50.7	6	68.0	4	59.0
..	7	48.9	4	63.0	4	52.0
..	6	54.0	4	56.0	4	60.0
..	7	55.7	4	62.5	3	66.7
..	6	50.0	4	48.5	3	68.0
..	5	48.4	4	48.5	4	53.0
..	7	48.9	4	52.5	4	56.0
..	6	44.3	4	63.5	3	62.7

of observations.
evidence of pollution.
evidence of intense pollution.

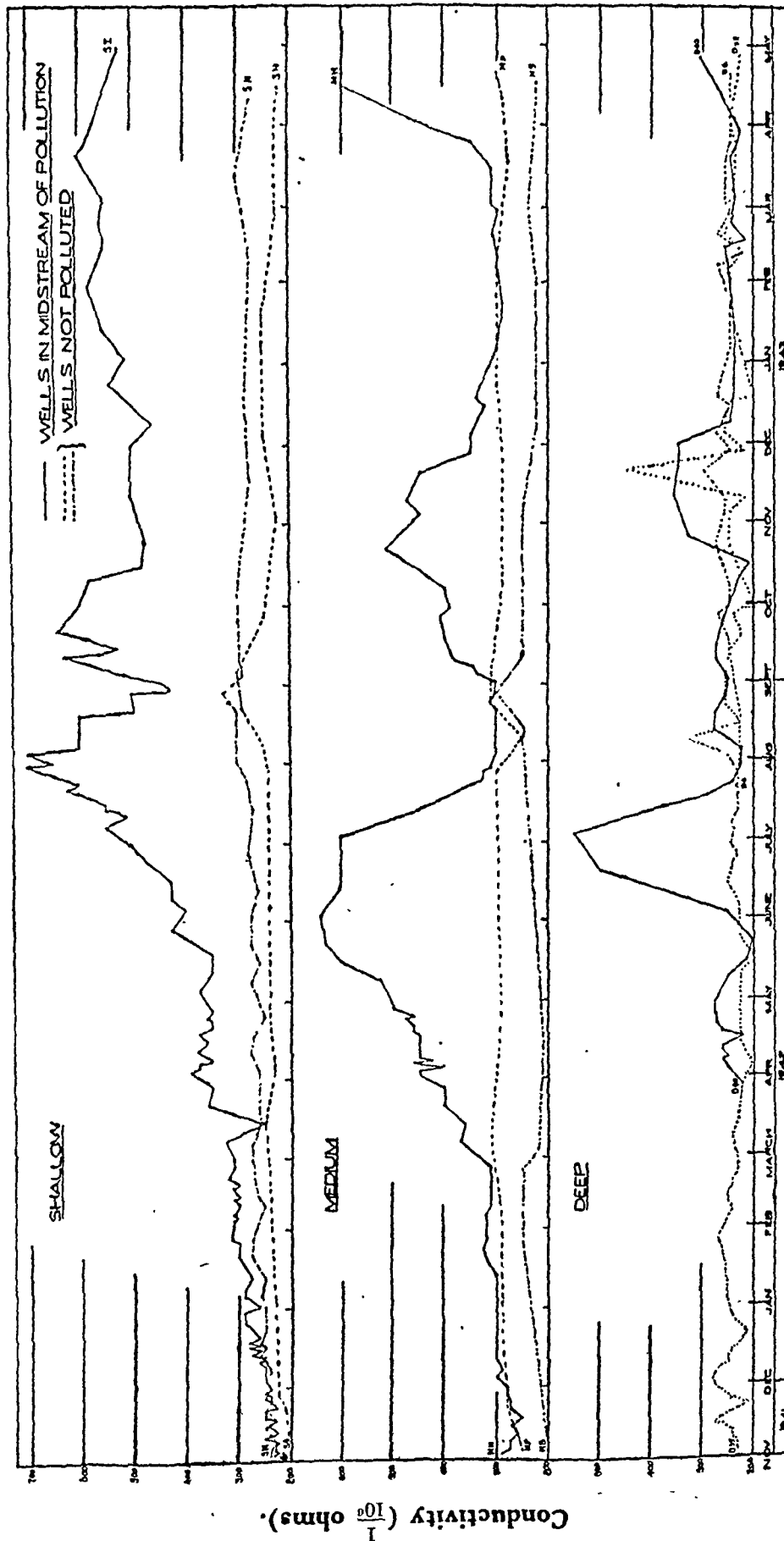


FIG. 6.—The trend of conductivity in polluted wells.

TABLE VIII.
Recovery of chemical pollution in shallow wells after seeding the latrine.

Distance in feet from latrine.	Wells.	Period.	pH.		Conductivity (1/10° ohms).		Acidity (ppt CaCO ₃).		Chlorides (ppt NaCl).		Alkalinity (ppm CaCO ₃).		Free ammonia (ppm NH ₃).		Albuminoid ammonia (ppm NH ₃).		Nitrates (ppm NO ₃).	
			N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
5	SM-SA.	Jan.-Apr. 1942	34	6.7	34	255.6	34	58.3	34	1.41	7	136.8	34	1.90	34	0.07	32	0.17
		May-Aug. 1942	25	6.6	25	275.6	25	58.5	25	1.78	25	185.4	2	2.23	2	0.08	25	0.08
		Sep.-Dec. 1942	17	6.6	17	265.4	17	53.6	17	1.63	16	161.9	17	0.08
		Jan.-Apr. 1943	16	6.6	16	261.3	16	56.2	16	1.59	16	191.5	16	0.14
5	SC-SK (in pollution stream).	Jan.-Apr. 1942	124	6.7	124	285.2	124	57.4	124	1.79	38	163.9	121	1.95	121	0.09	121	0.16
		May-Aug. 1942	100	6.6	100	403.6	100	83.0	99	4.04	100	235.1	36	3.25	35	0.21	98	0.10
		Sep.-Dec. 1942	72	6.6	72	397.3	72	90.1	72	3.55	71	218.8	32	3.15	32	0.19	72	0.10
		Jan.-Apr. 1943	54	6.6	54	378.9	54	80.3	54	3.01	54	243.9	13	3.12	13	0.15	52	0.15
15	SL ₁₇ -SL ₁₈	May-Aug. 1942	40	6.6	40	274.2	40	54.0	40	2.12	40	172.6	1	2.72	1	0.08	36	0.08
		Sep.-Dec. 1942	76	6.6	76	264.0	75	56.4	76	1.82	75	150.0	76	0.09
		Jan.-Apr. 1943	65	6.6	65	263.1	65	59.6	65	1.71	65	179.4	64	0.14
		Jan.-Apr. 1942	17	6.7	17	278.8	17	49.0	17	1.98	10	161.7	16	1.78	14	0.09	15	0.14
15	SL ₇ -SR ₁ (in pollution stream).	Jan.-Apr. 1942	114	6.7	114	292.2	114	46.4	114	2.32	113	179.3	14	1.87	14	0.13	114	0.11
		May-Aug. 1942	136	6.7	136	315.4	136	58.5	136	2.40	134	171.7	4	2.52	4	0.08	136	0.13
		Sep.-Dec. 1942	117	6.6	117	333.2	117	58.2	117	2.47	117	213.6	11	2.06	11	0.06	116	0.17
		Jan.-Apr. 1943	117	6.6	117	333.2	117	58.2	117	2.47	117	213.6	11	2.06	11	0.06	116	0.17

N indicates number of observations.

TABLE IX.

Recovery of chemical pollution in medium wells after seeding the latrine.

Distance in feet from latrine.	Wells.	Period.	pH.		Conductivity (1/10° ohms).		Acidity (ppm CaCO ₃).		Chlorides (ppht NaCl).		Alkalinity (ppm CaCO ₃).		Free ammonia (ppm NH ₃).		Albuminoid ammonia (ppm NH ₃).		Nitrates (ppm NO ₃).	
			N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
5	MN-MB.	Jan.-Apr. 1942	31	6.7	31	277.2	31	51.6	31	1.45	5	149.3	31	2.20	31	0.07	29	0.15
		May-Aug. 1942	16	6.6	16	289.3	16	54.5	16	1.73	16	183.6	16	0.07
		Sep.-Dec. 1942	16	6.6	16	278.1	16	55.3	16	1.68	16	175.8	15	0.09
		Jan.-Apr. 1943	16	6.6	16	273.1	16	53.8	16	1.72	16	179.5	16	0.19
	MD-ML (in pollution stream).	Jan.-Apr. 1942	97	6.7	97	306.2	97	64.5	97	1.82	24	181.2	95	2.30	95	0.10	96	0.16
		May-Aug. 1942	96	6.6	96	311.6	96	61.8	96	2.35	96	193.9	7	2.40	7	0.11	96	0.10
		Sep.-Dec. 1942	72	6.6	72	345.7	72	77.9	72	2.56	71	198.9	20	3.98	20	0.14	70	0.13
		Jan.-Apr. 1943	53	6.6	53	289.5	53	61.4	53	2.02	53	195.4	1	1.92	2	0.00	52	0.15
	MR ₃ -MR ₁	Jan.-Apr. 1942	7	6.8	7	298.3	7	43.2	7	1.61	4	164.0	7	2.01	7	0.09	7	0.13
		May-Aug. 1942	16	6.8	16	303.8	16	46.8	16	1.98	15	184.6	1	2.77	1	0.08	16	0.09
		Sep.-Dec. 1942	37	6.7	37	306.7	37	56.8	37	1.75	36	177.0	37	0.11
		Jan.-Apr. 1943	32	6.7	32	294.0	32	48.8	32	1.78	32	195.3	2	2.73	2	0.00	32	0.15
15	MC-ML ₁ (in pollution stream).	Jan.-Apr. 1942	11	6.7	11	290.5	11	45.8	11	1.67	7	173.7	9	1.92	9	0.10	10	0.12
		May-Aug. 1942	92	6.7	92	303.2	92	50.0	92	2.01	91	187.1	8	2.41	8	0.10	92	0.12
		Sep.-Dec. 1942	102	6.7	102	319.0	102	56.3	102	1.92	101	177.9	102	0.12
		Jan.-Apr. 1943	88	6.7	88	304.0	88	57.0	88	2.05	88	201.4	7	2.44	7	0.07	87	0.15
	M ₁ R ₂ -M ₁ L ₂	July-Sep. 1941	33	6.8	33	282.5	33	61.8	33	1.81	33	2.84	33	0.09
		Jan.-Apr. 1943	118	6.6	118	289.7	118	56.3	118	1.83	118	193.3	8	2.70	8	0.07	116	0.15
		July-Sep. 1941	26	6.7	26	287.6	26	68.9	26	1.75	26	3.08	26	0.08
		Jan.-Apr. 1943	9	6.6	9	280.0	9	57.3	9	2.13	9	178.7	9	2.68	9	0.17	8	0.16
	M ₂ R ₂ -M ₂ L ₁	July-Sep. 1941	26	6.7	26	287.6	26	68.9	26	1.75	26	3.08	26	0.08
		Jan.-Apr. 1943	9	6.6	9	280.0	9	57.3	9	2.13	9	178.7	9	2.68	9	0.17	8	0.16

N indicates number of observations.

TABLE X.

Recovery of chemical pollution in deep wells after seeding the latrine.

Distance in feet from latrine.	Wells.	Period.	pH.		Conductivity (1/10° ohms).		Acidity (ppm CaCO ₃).		Chlorides (ppht NaCl).		Alkalinity (ppm CaCO ₃).		Free ammonia (ppm NH ₃).		Albuminoid ammonia (ppm NH ₃).		Nitrates (ppm NO ₃).	
			N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
10	D ₈ -D ₁₃	{ Jan.-Apr. 1942	166	6.6	166	243.7	166	52.3	166	1.96	28	127.0	165	1.17	166	0.06	160	0.15
		{ May-Aug. 1942	147	6.6	147	253.8	147	56.3	147	2.18	147	163.1	8	0.95	7	0.06	142	0.08
		{ Sep.-Dec. 1942	208	6.7	208	252.1	208	57.5	208	1.79	204	147.6	6	1.10	5	0.52	203	0.09
		{ Jan.-Apr. 1943	184	6.6	184	248.3	184	57.9	183	1.76	184	168.5	181	0.14
	D ₁₀ -D ₁ (in pollution stream).	{ Jan.-Apr. 1942	137	6.6	137	240.8	137	54.3	137	2.36	58	121.6	136	0.81	134	0.06	135	0.13
		{ May-Aug. 1942	137	6.6	137	254.5	136	54.1	137	2.54	136	163.8	13	0.60	12	0.07	134	0.20
		{ Sep.-Dec. 1942	174	6.6	174	262.2	174	72.1	174	2.10	172	165.4	18	1.50	18	0.13	170	0.09
		{ Jan.-Apr. 1943	138	6.6	138	261.7	138	63.1	138	1.83	138	175.1	1	3.40	1	0.06	135	0.13
	DR ₅ -DL ₁	{ Jan.-Apr. 1942	4	6.6	4	230.0	4	46.7	4	2.04	1	140.0	4	0.59	4	0.09	4	0.07
		{ May-Aug. 1942	74	6.6	74	238.8	74	48.2	74	2.12	72	151.3	6	0.78	6	0.04	72	0.10
		{ Sep.-Dec. 1942	27	6.7	27	241.0	27	50.5	27	1.74	26	137.7	27	0.10
		{ Jan.-Apr. 1943	32	6.7	32	230.8	32	53.0	32	1.77	32	168.2	3	1.71	3	0.03	32	0.16
15	DL ₅ -DL ₁₀ (in pollution stream).	{ Jan.-Apr. 1942	5	6.7	5	220.2	5	29.7	5	2.44	5	132.7	3	0.77	3	0.06	5	0.14
		{ May-Aug. 1942	93	6.7	93	238.8	93	46.2	93	2.33	92	148.9	3	0.82	3	0.07	84	0.07
		{ Sep.-Dec. 1942	46	6.7	46	260.9	46	58.2	46	1.78	46	171.8	1	0.58	1	0.22	46	0.10
		{ Jan.-Apr. 1943	45	6.6	45	263.8	45	57.2	45	1.83	45	181.3	6	1.61	6	0.07	45	0.13
	{ DNR-DNL	{ Jan.-Apr. 1942	28	6.9	28	262.3	28	21.5	28	2.28	13	158.5	28	0.42	28	0.04	27	0.12
		{ May-Aug. 1942	31	6.9	31	285.3	31	23.3	31	2.57	30	167.0	2	0.44	2	0.10	30	0.07
		{ Sep.-Dec. 1942	12	6.9	12	266.7	12	29.5	12	2.12	12	159.0	12	0.08
		{ Jan.-Apr. 1943	19	6.9	19	277.6	19	32.6	19	2.36	19	177.7	5	0.37	5	0.06	19	0.13
	{ D ₁ R ₅ -D ₁ L ₅	{ July-Sep. 1941	32	6.7	32	224.2	32	48.2	32	1.79	32	1.06	32	0.06	..	0.15
		{ Jan.-Apr. 1943	10	6.6	10	221.1	10	33.5	10	2.02	10	144.4	8	1.95	8	0.19	9	0.15
	{ D ₂ R ₅ -D ₂ L ₄	{ July-Sep. 1941	29	6.7	29	228.9	29	52.8	29	1.71	29	1.23	29	0.08	..	0.16
		{ Jan.-Apr. 1943	9	6.6	9	227.5	9	53.0	9	2.05	9	149.5	9	1.03	9	0.04	9	0.16
50	{ D ₂ R ₅ -D ₂ L ₄	{ July-Sep. 1941	20	6.7	20	245.9	20	60.1	20	1.74	20	1.41	20	0.09	..	0.19
		{ Jan.-Apr. 1943	8	6.6	8	241.4	8	55.4	8	2.21	8	157.7	8	1.50	8	0.04	8	0.19

N indicates number of observations.

TABLE XI.

Extent of flow of chemical pollution in wells.

Depth of strata.	Date of first appearance.	Wells along which chemical pollution flowed.	Wells showing intense pollution.	Extent of flow in feet.
Shallow ..	Wells SG and SI in 5' zone Jan.-Apr. 1942.	Along wells SC to SK (5' zone) to SR ₁ to SL ₇ (15' zone).	SG and SI	At least 15
Medium ..	Jan.-Apr. 1942	Along wells MD to ML (5' zone) to MC to ML ₁₀ (15' zone).	MH	Between 15 and 25
Deep ..	May-Aug. 1942	Along wells D ₃₆ to D ₄ (10' zone) to DL ₂ to DL ₁₀ (15' zone).	D ₂₇ and D ₄₁	Between 15 and 25

It may be observed that the flow of chemical products from the latrine was confined to the stream of flow indicated by the salt experiment (Dyer and Bhaskaran, 1943a). These constituents were detectable to a distance of 15 feet beyond which point they were too dilute to be distinguishable from the natural content of the surrounding ground water. Pollution flowed through the shallow wells more intensely than either the deep or medium wells. The non-recovery of the products in the D_n wells indicates that there was probably little significant flow into the deeper layers. It is of interest to note that under conditions of intensive use of a village well, simulated by pumping 600 gallons each morning, pollution was not recovered beyond 15 feet from the latrine.

Experiments were carried out to determine the nature of the nitrogenous products flowing out of the latrine and the extent to which these accounted for the pollution recovered in the wells. Parallel samples from representative polluted wells, control wells and the latrine were examined for the different forms of nitrogens—total organic, ammonia, nitrites and nitrates. The data are presented in Table XII :—

TABLE XII.

Nature of the nitrogenous products recovered from wells.

Depth of strata.	Well.	NITROGEN AS PPM NH ₃ .			
		Total.	Organic.	Ammonia.	Nitrites and nitrates.
Shallow ..	Polluted SG	5.28	1.44	3.80	Less than 0.1
	„ SI	3.84	1.48	2.36	do.
	Control XSC	2.95	0.27	2.68	do.
Medium ..	Polluted MH	4.27	0.27	4.00	Less than 0.1
	Control XM ₁ C	3.73	0.21	3.52	do.
Deep ..	Polluted D ₃₆	3.85	1.17	2.68	Less than 0.1
	„ D ₃₇	3.39	1.79	1.60	do.
	Control D ₃ C	3.23	0.21	3.02	do.

The results show that nitrogen which flowed out of the latrine was mainly in the form of ammonia and that nitrogenous products accounted for only a fraction of the increase in soluble minerals recovered from the polluted wells.

Effect of zones and seasons.—The wells above the latrine were observed throughout the period of the investigation and the data collected furnish a basis of comparison with those obtained from wells below the latrine after seeding was begun. Conductivity and acidity were used as indicators of possible chemical pollution. The period was subdivided into intervals of four months with from 1 to 12 examinations of the individual wells in any interval. The mean value for each well and interval are given in Tables XIII and XIV :—

TABLE XIII.

Conductivity (1/10⁶ ohms) of water samples in wells 25 feet above the latrine observed throughout the period.

Depth of strata.	Wells.	1941.				1942.				1943.				
		May-Aug.		Sep.-Dec.		Jan.-Apr.		May-Aug.		Sep.-Dec.		Jan.-Apr.		
		N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	
Shallow	..	XSR ₂	3	283.3	4	241.2	6	255.8	3	233.3	3	250.0	5	260.0
		XSR ₁	3	265.0	1	190.0	2	230.0	9	247.8	4	252.5	5	253.0
		XSC	3	265.0	1	220.0	2	235.0	2	275.0	2	262.5	4	258.8
		XSL ₁	3	263.3	2	255.0	2	240.0	1	300.0	4	257.5	5	278.0
		XSL ₂	3	350.0	6	230.0	2	245.0	12	224.2	4	250.0	5	256.0
Medium	..	XMR ₂	2	305.0	3	321.7	5	327.0	1	325.0	3	315.0	6	326.7
		XMR ₁	4	313.8	2	337.5	2	350.0	1	325.0	4	313.8	5	331.0
		XML ₁	3	300.0	1	300.0	2	327.5	2	322.5	3	318.3	5	327.0
		XML ₂	5	264.0	2	250.0	2	305.0	2	282.5	2	290.0	5	310.0
Deep	...	XDR ₂	3	253.3	4	261.2	2	277.5	1	275.0	4	293.8	5	286.0
		XDR ₁	3	241.7	3	250.0	1	250.0	10	249.0	4	263.8	5	271.0
		XDC	3	265.0	3	278.3	5	279.0	2	272.5	3	283.2	5	279.0
		XDL ₁	3	251.7	1	240.0	3	250.0	2	250.0	3	288.3	4	283.8
		XDL ₂	3	246.7	2	237.5	1	240.0	3	255.0	3	261.7	5	252.0

N indicates number of observations.

TABLE XIV.

Acidity (ppm CaCO₃) in water samples in wells 25 feet above the latrine observed throughout the period.

		1941.				1942.				1943.				
Depth of strata.	Wells.	May-Aug.		Sep.-Dec.		Jan.-Apr.		May-Aug.		Sep.-Dec.		Jan.-Apr.		
		N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	
Shallow	..	XSR ₂	3	93.7	4	73.6	6	62.7	3	48.0	3	67.3	5	71.2
		XSR ₁	3	65.7	1	60.0	2	47.0	9	52.2	4	58.0	5	60.8
		XSC	3	76.3	1	64.0	2	45.0	2	54.0	2	70.0	4	62.0
		XSL ₁	3	71.0	2	63.0	2	57.0	1	50.0	4	61.5	5	65.6
		XSL ₂	3	94.0	6	68.8	2	51.0	12	48.3	4	63.0	5	55.2
Medium	..	XMR ₂	2	68.0	3	67.3	5	66.4	1	60.0	3	43.3	6	54.7
		XMR ₁	4	84.0	2	91.0	2	74.0	1	70.0	4	64.0	5	73.6
		XML ₁	3	78.7	1	78.0	2	70.0	2	62.0	3	62.0	5	74.4
		XML ₂	5	65.4	2	56.0	2	60.0	2	54.0	2	66.0	5	64.0
Deep	..	XDR ₂	3	60.3	4	76.0	2	66.0	1	56.0	4	76.0	5	66.4
		XDR ₁	3	45.7	3	51.3	1	26.0	10	39.6	4	43.5	5	52.0
		XDC	3	54.0	3	63.3	5	62.0	2	59.0	3	83.3	5	64.0
		XDL ₁	3	62.7	1	44.0	3	40.7	2	41.0	3	59.3	4	59.0
		XDL ₂	3	62.0	2	79.0	1	60.0	3	61.3	3	71.3	5	83.2

N indicates number of observations.

Tables XIII and XIV show that distinct well-to-well differences occurred and in some instances were quite pronounced. Thus, conductivity in well XDR₁ was consistently less than that in its neighbour XDC, and that in XML₂ was less than that in well XML₁ or XMR₁. Acidity in adjoining wells XDR₁ and XDR₂, XMR₁ and XMR₂, XSR₁ and XSC showed marked variations.

Observations of wells against the direction of flow were less frequent and it was not possible to see the seasonal trend over short periods. For the period as a whole conductivity and acidity varied considerably in wells in shallow and medium strata. Conductivity increased in shallow wells in 1942 and 1943. In medium strata there was a rise in January-April 1942, followed by a decrease the rest of the year. There was a general tendency for acidity values to decrease both in the shallow and medium strata throughout the period.

The means of the conductivity and acidity values for wells in the 5- and 25-foot zones above the latrine are shown by season in Table XV :—

TABLE XV*.

Chemical analyses of water samples from wells above the latrine observed throughout the period.

Mean conductivity (1/10⁶ ohms).

Depth of strata.	Distance in feet above latrine.	Number of wells.	N	1941.		1942.			1943.
				May-Aug.	Sep.-Dec.	Jan.-Apr.	May-Aug.	Sep.-Dec.	Jan.-Apr.
Shallow .. {	5	4	129	274.4	230.0	255.6	275.6	265.4	261.3
	25	5	111	265.3	227.2	241.2	256.1	254.5	261.2
Medium .. {	5	4	114	274.4	245.2	277.2	289.3	278.1	273.1
	25	4	72	295.7	302.3	327.4	313.8	309.3	323.7
	50	5	108	280.5	301.4	319.5	299.3	308.9	312.1
Deep .. {	10	18	232	243.1	242.5	245.4	256.3	247.5	249.8
	25	5	99	251.7	253.4	259.3	260.3	278.2	274.4
	50	6	111	252.2	263.2	264.8	247.2	267.3	267.6

Mean acidity (ppm CaCO₃).

Shallow .. {	5	4	129	79.5	64.2	58.3	58.5	53.6	56.2
	25	5	111	80.1	65.8	52.5	50.5	64.0	63.0
Medium .. {	5	4	114	74.1	66.0	51.6	51.5	55.3	53.8
	25	4	72	74.0	73.1	67.6	61.5	58.8	66.7
	50	5	108	62.2	84.9	60.6	56.6	58.4	61.6
Deep .. {	10	18	232	64.9	63.8	50.9	60.1	58.6	60.0
	25	5	99	56.9	62.7	50.9	51.4	66.7	64.9
	50	6	111	72.0	72.1	64.6	62.3	69.3	62.5

N indicates number of observations.

* In view of the fact that the number of observations was more or less the same for all wells in each period, it was considered that this method of estimation of the general behaviour of wells would prove quite adequate.

The seasonal variation was not the same in the different zones. Analysis showed that interaction between zones and seasons was significant for conductivity in the medium strata. Differences in acidity values were less pronounced.

Biochemical oxygen demand (B. O. D.).—Since there was no dissolved oxygen in the ground water a 5-day test for B. O. D. was used in an effort to trace pollution. The results varied from 0 to 15 parts per million (ppm) prior to seeding and in wells above the latrine observed throughout the period. Large and varying amounts of iron partly accounted for the high values. Means for wells 25 and 50 feet above the latrine for each period are presented in Table XVI. The zone and depth differences were not very significant. During the interval January–April 1942, mean B. O. D. rose in all depths and zones and continued to rise somewhat during the remainder of the period. In view of the large error involved in the experimental technique a mean above 15 ppm may be indicative of pollution.

TABLE XVI.

Mean biochemical oxygen demand (5-day test for B. O. D. in ppm) in water samples from wells above the latrine observed throughout the period.

Distance in feet above latrine.	Depth of strata.	1941.		1942.				1943.			
		Sep.-Dec.		Jan.-Apr.		May-Aug.		Sep.-Dec.		Jan.-Apr.	
		N	Mean	N	Mean	N	Mean	N	Mean	N	Mean
25	Shallow	8	3.5	8	6.6	18	8.0	8	7.5	2	5.7
	Medium	6	4.4	4	7.2	2	9.6	4	12.0	2	10.8
	Deep	6	3.2	4	4.4	7	6.4	7	6.5	4	8.2
50	Medium	3	2.6	6	5.9	10	12.0	6	8.1	1	6.6
	Deep	2	1.2	2	5.1	16	7.4	6	7.4	3	7.0

N indicates number of observations.

After the latrine was seeded over 2,000 samples from wells were given the 5-day test for B. O. D. and the data for the different periods are given in Table XVII. Mean values rose to a height of 18.0 ppm in wells of the 5-foot zone in shallow and medium strata known to be in the pollution stream. The values were erratic, however, as shown by relatively high values for wells not known to be polluted from evidence of other tests. B. O. D. did not prove to be a satisfactory indicator in this area.

TABLE XVII.

Organic matter in wells as shown by biochemical oxygen demand (5-day test for B. O. D. in ppm) after seeding the latrine.

Depth of strata.	Distance in feet from latrine.	Wells.	5-DAY B. O. D. IN PPM.										
			1941.		1942.				1943.				
			Nov.-Dec.		Jan.-Apr.		May-Aug.		Sep.-Dec.		Jan.-Apr.		
			N	Mean	N	Mean	N	Mean	N	Mean	N	Mean	
Shallow.	5 In pollution stream.	SM-SA	12	5.5	11	4.8	18	9.0	16	6.4	6	6.1	
		SC	2	14.6	4	5.7	16	10.2	12	6.3	3	8.	
		SE	15	5.1	8	9.9	15	7.7	10	6.6	3	1.0	
		SG	17	4.5	24	18.0	15	16.4	9	7.9	4	6.6	
		SI	16	4.3	16	11.1	18	11.4	11	10.8	4	11.6	
		SK	18	4.7	6	2.3	16	8.1	9	7.1	3	6.0	
	15	SL ₅ -SL ₁₅	9	11.1	33	5.7	19	5.2	
		SR ₅ -SL ₇	6	2.1	12	8.6	32	9.7	67	7.1	32	4.8	
	Medium.	5 In pollution stream.	MN-MB	17	2.2	12	8.5	11	12.6	15	5.3	7	9.2
			MD	2	2.7	14	7.1	10	5.8	1	0.0
			MF	7	4.6	7	6.3	9	9.6	10	13.2	1	3.0
			MH	21	5.6	17	17.6	18	17.0	11	13.9	2	11.1
			MJ	16	4.5	7	2.6	14	8.4	11	14.1	3	4.2
			ML	16	3.7	8	6.1	16	10.6	9	7.1	2	3.9
15		MR ₅ -MR ₇	2	0.0	6	14.6	18	5.1	7	12.0	
		MC-ML ₇	2	2.7	4	7.5	26	11.0	47	7.4	24	6.2	
Deep.		10	D ₅ -D ₂₅	76	4.0	56	3.6	97	7.5	130	7.2	58	4.6
			D ₃₅ -D ₄	40	4.8	41	6.1	95	8.0	82	8.4	41	6.4
	15	DR-DL ₂	5	2.0	1	0.6	14	8.0	2	11.4	
		DL ₂ -DL ₁₀	3	6.0	15	10.4	4	8.7	

N indicates number of observations.

Italicized figures indicate evidence of pollution.

Antique figures indicate evidence of intense pollution.

Bacterial pollution prior to seeding.—The flow of bacteria from the latrine was easily traced at Singur because of the low content of the ground water in its natural state. Many wells gave negative results with dilutions of 100 c.c. The analysis of water samples was by the dilution procedure with quantities varying from 100 c.c. to less than 0.1 c.c. of water when the number of gas-formers was high. Parallel samples were plated on Endo medium and the poured plates incubated at 37°C, and counted for *coliform* reds at the end of 48 hours. The most probable number (M. P. N.) of gas-formers was computed from the total size of sample and the number and dilution of the tubes showing acid and gas by the method of Thomas (*loc. cit.*).

Due to the small number of gas-formers in the water prior to seeding differences between well depths and zones were not significant and the data for all wells were pooled for each round. The computed M. P. Ns. with standard errors are given in Table XVIII:—

TABLE XVIII.

Most probable number of gas-formers per 1,000 c.c. of water prior to seeding the latrine.

Round.	M. P. N.	Standard error.
1st (1-7-41 to 16-7-41)	6.3	± 0.6
2nd (24-7-41 to 7-8-41)	3.2	± 0.4
3rd (4-9-41 to 19-9-41)	8.3	± 0.6

Table XVIII indicates that the frequency varied from 3.2 to 8.3 gas-formers per 1,000 c.c. of water during the three rounds. The difference is probably significant but is not important in view of the small number of gas-formers present.

Results of the bacteriological examination of wells against the direction of flow from the bore-hole latrine for the different periods are presented in Table XIX. There was a tendency for the frequencies to decrease in 1942 and 1943 as compared with those for 1941. These changes are not important, however, since the maximum number of gas-formers at any season was small.

The results of the differential tests of the cultures isolated from the samples are presented in Table XX. They were run on all the wells located against the direction of ground-water flow from the latrine during 1941–1943. The results show that 80 per cent of the cultures were definitely non-faecal in origin and that only one out of 128 cultures could be considered as faecal *coli*.

Bacterial pollution following seeding.—The most probable numbers of gas-formers for different wells and groups of wells for periods following the seeding of the latrine are given in Tables XXI and XXII. They show conspicuous pollution in wells in the 5-foot zone. Means for wells MF and SK were high at one time or another and well SI gave a high frequency of gas-formers for all three periods in 1942. *B. coli* density in this well (SI) rose to 100 per c.c. on the 30th June, 1942, and continued high through July. Later, the numbers decreased and virtually no gas-formers were present in 1943.

In the 10-foot zone *B. coli* were recovered in wells D₅ and D₂₁. The number of organisms was small in well D₅ but very high in well D₂₁ over a short period. It is difficult to explain the behaviour of this well, however, since no chemical products of pollution were recovered during the period (except for a slowly rising acidity) and it lay within the range of the non-polluted wells. It should be noted, however, that capsules of chrysomias larvæ were recovered from this well on 3rd August, 1942.

TABLE. XIX.

The most probable number (M. P. N.) of gas-formers per 1,000 c.c. of water in samples from wells above the latrine observed throughout the period.

Depth of strata.	Distance in feet above latrine.	Wells.	1941.	1942.		1943.
			July.-Dec.	Jan.-June.	July-Dec.	Jan.-Apr.
Shallow ..	25	XSR ₂	—	—	—	—
		XSR ₁	33	13	8	—
		XSC	6	—	—	—
		XSL ₁	10	4	1	—
		XSL ₂	5	—	—	—
Medium ..	25	XMR ₂	10	—	—	—
		XMR ₁	—	—	—	—
		XML ₁	12	—	—	—
		XML ₂	—	—	—	16
	50	XM ₁ R ₂	—	—	1	—
		XM ₁ R ₁	2	—	—	—
		XM ₁ C	2	—	17	—
		XM ₁ L ₁	2	—	—	2
		XM ₁ L ₂	—	1	—	1
Deep ..	25	XDR ₂	2	—	1	—
		XDR ₁	2	1	2	—
		XDC	2	—	—	—
		XDL ₁	3	—	2	1
		XDL ₂	—	—	—	—
	50	XD ₁ R ₁	5	—	—	—
		XD ₁ R ₂	—	—	—	—
		XD ₁ R ₁	21	11	—	—
		XD L	—	—	2	—
		XD ₁ L ₂	—	7	6	—
XD L ₁		—	—	14	—	
AVERAGE ..			6	5	5	2

— indicates less than 1 organism per 1,000 c.c. of sample.

TABLE XX.

Nature of gas-formers isolated from wells against the direction of ground-water flow observed throughout the period.

Type of organism.	1941.		1942.		1943.		Total.	Origin.		
	July.-Dec.		Jan.-June.		July-Dec.				Jan.-Apr.	
<i>B. coli</i> I	1	..	4	..	5*	Fæcal.				
<i>B. coli</i> II	2	..	1	3	22	Doubtful.				
Irregular lactose fermenters	11	1	4	..						
Mixed (Intermediate, ærogenes and others)	31	7	27	3	105	Non-fæcal.				
Anærobcs	8	1	5	4						
Soil	9	..	9	1						
TOTAL ..	62	9	50	11	132					

* Of these 5 cultures, 4 were traced as due to external contamination and the fifth showed a P. N. of 23 organisms in 1,000 c.c. of water.

TABLE XXI.

Recovery of gas-formers from wells (means M. P. N. per 1,000 c.c. of water sample) after seeding the latrine.

Depth of strata.	Distance in feet from latrine.	Wells.	1941.		1942.		1943.
			Nov.-Dec.	Jan.-Apr.	May-Aug.	Sep.-Dec.	Jan.-Apr.
Shallow ..	5	SM-SA	*	*	21±3.4	2±1.0	2±0.9
		SC	14±4.8	1	18	16±4.7	..
		SE	1	2	1	1	9
		SG	1	3	13	1	*
		SI	1	13±2.2	84±8.5	10±3.5	*
		SK	2	*	20	11±4.0	*
	15	SL ₁₀ -SL ₅	15±2.7	4±0.9	2±0.7
		SL ₇ -SR ₂	*	*	1	2	*

* represents all tubes negative.

TABLE XXI--concl'd.

Depth of strata.	Distance in feet from latrine.	Wells.	1941.	1942.			1943.
			Nov.-Dec.	Jan.-Apr.	May-Aug.	Sep.-Dec.	Jan.-Apr.
Medium ..	5	MN-MB	5±1.3	1±0.5	12±2.7	8±2.0	1±0.5
		MD	*	5	9	8	*
		MP	2	34±11.9	6	9±3.5	*
		MH	3	1	9	8	*
		MJ	2	7	5	6	*
		ML	2	*	*	3	*
	15	MR ₃ -MR ₁	14±3.9	*	*	12±2.4	*
		MC-ML ₇	2	20	20	7	1
	25	M ₁ R ₃ -M ₁ L ₅	*
	35	M ₂ R ₁ -M ₂ L ₁	1
Deep ..	10	D ₃ -D ₃₅	5±0.5	10±1.0	4±0.6	3±0.5	2±0.4
		D ₃₆ -D ₄	6	3	4	3	*
	15	DR ₃ -DL ₁	*	*	5±0.9	*	1±0.7
		DL ₅ -DL ₁₆	3	2
	20	D _n R-D _n L	..	20	3	4	1
	25	D ₁ R ₅ -D ₁ L ₅	2
	35	D ₂ R ₁ -D ₂ L ₁	*
	50	D ₃ R ₃ -D ₃ L ₃	*
	Wells showing <i>B. coli</i>		..	SI	SI		D ₅
				SK D ₅	MF D ₂₁		
			MF D ₂₁	D ₂₁			

* represents all tubes negative.

TABLE XXII.

Most probable number of coliform organisms in wells showing B. coli after seeding the latrine.

Depth of strata.	Distance in feet from latrine.	Well.	Date of sample.	M. P. N. IN 1,000 C.C. SAMPLE.	
				Dilution method.	Plate counts in Endo.
Shallow ..	5	SI	28-3-42	259	100,000 30,000
			30-6-42	3,363 +	
			8-7-42	3,363 +	
			22-7-42	3,363 +	
			24-7-42	3,363 +	
			1-8-42	3,363	
			5-8-42	3,363	
			7-8-42	41	
			21-8-42	20	
			1-9-42	195	
		SK	9-7-42	3,363 +	
			13-8-42	66	
Medium ..	5	MF	6-7-42	259	
			11-9-42	259	
Deep ..	10	D ₆	5-8-42	23	
			7-1-43	44	
		D ₂₁	31-8-42	2,274 +	
			12-9-42	3,363 +	
			30-9-42	2,274	

Bacterial pollution travelled a distance of 10 feet although only a few *B. coli* were recovered in only one of 13 chemically polluted wells in that zone. With the formation of the latrine defences *B. coli* were recovered less frequently and in smaller numbers. Intensity of bacterial pollution was greater in the shallow wells.

Recovery of chrysomia larvæ in wells.—During the analysis of water samples from the wells an interesting observation was made. Chrysomia larvæ and their capsules which were present in the latrine soon after seeding began were recovered from some of the 5- and 10-foot zone wells. The observations are summarized in Table XXIII.

TABLE XXIII.

*Recovery of chrysomia larvæ and capsules from wells
after seeding the latrine.*

Depth of strata.	Distance in feet from latrine.	Wells.	Date.	Number of larvæ.	Number of capsules.
Shallow ..	5	SO	5-8-42	Nil	5
		SO	19-8-42	Nil	3
		SO	1-9-42	5	6
		SC	12-8-42	Nil	1
		SC	26-8-42	Nil	3
		SC	1-9-42	3	4
		SC	9-9-42	Nil	2
		SI	14-8-42	Nil	2
		SI	21-8-42	Nil	2
		SI	1-9-42	Nil	3
		SI	4-9-42	4	0
		SM	14-8-42	Nil	2
		SM	4-9-42	Nil	4
		SE	15-8-42	Nil	2
		SE	5-9-42	Nil	3
		SG	24-8-42	Nil	1
		SG	5-9-42	2	2
		SQ	29-8-42	Nil	3
		SA	5-9-42	2	20
Medium ..	5	MP	10-8-42	Nil	2
		MF	14-8-42	Nil	1
		MF	21-8-42	Nil	3
		MF	4-9-42	4	12
		ML	19-8-42	Nil	1
		ML	1-9-42	1	1
		ML	9-9-42	Nil	3
		MH	24-8-42	Nil	1
		MH	9-9-42	Nil	3
		MJ	5-9-42	3	5
Deep ..	10	D21	3-8-42	Nil	2
		D39	4-9-42	Nil	4

These larvæ were recovered not only in wells in the direction of ground-water flow but in wells from all sections of the circles. In spite of the presence of the larvæ it is interesting to note that many of these wells did not show any bacterial pollution.

Examination of the well strainers at the end of the experiment revealed that in some instances the screens contained holes large enough to permit larvæ from the latrine to enter the well. Eggs also may have been carried along the stream of flow and may have hatched near the well thus allowing the capsules to enter.

DISCUSSION.

The present inquiry has revealed a naturally high standard of purity of the ground water at the site of the experiment in spite of the shallow depths at which it was tapped. Lactose fermenters were few (from 3 to 8 organisms per 1,000 c.c. of water) and were for the most part of non-fæcal origin. *B. coli* were virtually absent. The bacteriological purity of the water was little affected by seasonal and other environmental factors although the number of gas-formers decreased somewhat during the period.

Chemical analyses showed a low organic-matter content of the water in its natural state. This was indicated by the amount of albuminoid ammonia present. pH did not vary and the water was well buffered by bicarbonates. Minerals were present in only small amounts and were largely accounted for by carbonates and chlorides. Nitrites and nitrates were within limits.

Chemical constituents were affected by seasonal and environmental factors and the presence of pollution had to be interpreted in the light of these variations in the natural state of the water. Differences associated with depth were highly significant and those due to zone and period were also marked. There was even a well-to-well variation in the same zone and depth. Although seasonal trends were marked there were no conspicuous differences between those of the different zones.

The high bacterial purity of the water continued over the two years, in wells above the latrine, although the water table rose to as high as 3 feet below the ground surface.

These observations indicate that shallow wells may not be dangerous from the hygienic point of view in this type of soil. Shallow wells, properly protected from surface pollution, may constitute the solution of the water-supply problem in rural India. The high cost of deep wells makes their use prohibitive and, furthermore, shallow wells have a lower mineral content than is found in the deep wells of the plains of India.

The other important finding in this investigation was the limited extent of the pollution stream entering the ground water from a bore-hole latrine. In a soil medium of sand as coarse as 0.2 mm. effective size and 40 per cent pore space and with a ground-water slope of 1 in 200, bacterial pollution did not travel more than 10 feet from the latrine.

In judging pollution, the state of the ground water before the latrine was seeded together with the seasonal changes revealed by samples from wells against the direction of flow were used as a basis for comparison with changes occurring in wells lying within the stream of pollution flowing from the latrine.

When water is withdrawn from a well, surrounding soil water-flows in to re-establish equilibrium. The distance affected depends on the amount of water withdrawn, the rate of withdrawal and the character of the soil, and this area is known as the circle of influence. By field experiment its radius was found to be about 20 feet at this site and the D_n pumps were placed so that the latrine was within this range. By pumping 600 gallons each morning before sampling, a flow of soil water similar to that induced by the use of a rural village well was effected.

Gross *B. coli* pollution existed to a distance of 5 feet from the latrine and only one out of 13 wells in the stream flow showed mild *B. coli* pollution in the 10-foot zone. The flow of *B. coli* occurred after septic action was set up in the latrine and was intensive over a period of two months (July-August 1942). Later this organism was recovered less frequently and eventually disappeared showing its limited dispersion in the soil. Shallow wells were more intensely polluted than the medium or deep wells in spite of the high concentration of *B. coli* at the bottom of the latrine.

Organic matter indicated by the rise in biochemical oxygen demand also flowed a distance of 5 feet in a manner similar to bacteriological pollution except that it was recovered in more wells. Lactose fermenters plus a rise in B. O. D. were always accompanied by intense chemical pollution.

Chemical products flowed farther than bacterial and colloidal organic matter and were traced to 15 feet before becoming too dilute to be distinguishable. Unlike bacteria, chemical products flowed as a continuous belt along the stream with high intensity in the mid-stream wells. Shallow wells also showed more chemical pollution than either medium or deep wells. Apparently the flow of pollution is confined to the more fluid regions of the latrine and there is little flow downward into the deeper strata.

The picture revealed by the pollution recovery in wells is similar to that furnished by the flow of water through a sand-filter bed. In addition it gives insight into the processes *in situ* under the surface of the ground. Due to the ground-water slope, products from the more fluid regions of the latrine are carried along the stream and are filtered as they pass through the soil. The extent of flow of bacteria depends on the efficiency of the filtration process. The amount of chemical products that emerge is small and eventually becomes too dilute to be distinguishable.

Study of the changes in the bore-hole latrine showed that only a fraction of the added excreta was disintegrated and that the septic process proceeded only to the ammonia stage. The life of a bore-hole latrine may be extended by removing the sludge as is done with a septic tank. In this case there may be an increase in the flow of bacteria for a time, but the same defence principle will operate and pollution will travel little if any farther than it did in the first instance. The situation will be similar if a new latrine is driven in the neighbourhood of the original one since the defence mechanism has already been established.

SUGGESTIONS FOR CHOOSING A SITE FOR A BORE-HOLE LATRINE IN PROXIMITY TO A WELL.

The flow of ground water will follow roughly the slope of the land especially if the dip is toward a stream bed. The following suggestions are offered regarding the choice of a site for a bore-hole latrine:—

1. If space permits, the latrine should be placed on the slope below the well.
2. If the latrine must be placed above the well, a hole should be put down to ascertain the type of soil at a depth equal to that of the proposed latrine.
3. If soil at that depth is composed of sand with an effective size of 0.2 mm. or less, the latrine may be placed as close as 20 feet to the well.
4. If the sand is as coarse as 0.3 mm. effective size, the latrine should be placed 50 feet away.
5. If sand coarser than 0.3 mm. effective size is found, a careful study should be made before the latrine is placed.
6. If the soil is composed of clay, a distance of 20 feet is sufficient.

The only equipment necessary for obtaining soil samples is a post-hole auger and three standard sieves with meshes from 0.179 mm. to 0.35 mm.

SUMMARY.

An investigation of the nature and extent of pollution flowing from a bore-hole latrine into the ground water was made at a site in West Bengal, India, during the period from September 1940 through April 1943. Preliminary studies were made of soil conditions, and the direction of ground-water flow and velocity were determined before the experimental field was completed and routine sampling of wells begun. The results of the investigation may be summarized as follows:—

A. Natural status of the ground water prior to seeding of the latrine:—

1. Bacteriological studies showed a concentration of lactose fermenters of from 3 to 8 per 1,000 c.c. of water, almost wholly of non-faecal origin.

2. Chemical analyses were made for pH, conductivity, chlorides, acidity, different forms of ammonia and B. O. D. Chemical constituents of the water varied significantly at different depths, zones and seasons.

3. Water samples from wells above the latrine and from those not in the immediate direction of flow were examined at intervals throughout the period and the results used as an indication of the normal state of the ground water.

B. Pollution flow following seeding of the latrine :—

1. The latrine was seeded with two gallons of night-soil on 330 days between November 1941 and April 1943. Septic action began in March 1942, and continued thereafter with undiminished vigour. The septic process only reached the ammonia stage. Only a fraction of the excreta added was disintegrated and at the close of the experiment there was a 2-foot column of sludge in the latrine.

2. Routine observations were made of meteorological and ground-water conditions. Annual rainfall ranged from 40 to 80 inches, and the maximum slope of the water-table was 1 in 200. Effect of seasonal changes on biological decomposition in the ground water was not pronounced.

3. Experiments on the true and false water-table near the latrine showed that the differences were not large enough to affect the flow of pollution.

4. Six hundred gallons of water were pumped daily from wells situated 20 feet from the latrine in the direction of flow to simulate conditions of use of a village well.

5. Bacteriological pollution was recovered in wells 10 feet from the latrine with large numbers of *B. coli* found in wells in the 5-foot zone over a period of two months. Numbers diminished later and the organism was virtually absent during the final period.

6. Chemical pollution flowed with the stream to a distance of 15 feet. It was more intense in shallow than in medium or deep wells and was characterized by increase in conductivity, alkalinity, chlorides, acidity and ammonias. Nitrogenous products accounted for only a fraction of the pollution recovered.

7. Organic matter indicated by the 5-day test for B. O. D. was recovered in the 5-foot zone. It was probably colloidal in nature and was filtered out beyond that point.

8. Conductivity proved to be the most sensitive and hence the most satisfactory test for tracing chemical pollution.

9. Chrysomia larvæ and their capsules were recovered from wells in the 5- and 10-foot zones where screens were damaged. They were found not only in wells in the direction of flow but all around the latrine.

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STUDIES ON THE DESTRUCTION OF VITAMIN A IN SHARK-LIVER OIL.

Part III.

RELATION BETWEEN VITAMIN A DESTRUCTION AND PEROXIDE NUMBER.

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FRIDERICIA (1924) and Powick (1925) established that rancid fats destroyed vitamin A and suggested that the destruction might be due to organic peroxides. This theory has been confirmed by subsequent work (Rosenheim and Webster, 1926; Wokes and Willimott, 1927; Whipple, 1936; Lowen *et al.*, 1937; Smith, 1939). Further, Simons *et al.* (1940) found that the percentage of vitamin A oxidized in fish-liver oils at various peroxide values was independent of the initial concentration of the vitamin. Lease *et al.* (1938) observed that the destruction of the vitamin in rancid fats by peroxides would continue within the stomach of the rat during the bio-assay. In this paper the relation between the peroxide number and the vitamin A destroyed in shark-liver oil when exposed to air in thin layers at different temperatures has been studied.

In the oxidation of fats and oils exposed to air, if the peroxide number is plotted against time, a curve of sinuous character is obtained which is characteristic of an autocatalytic reaction. During the first period (induction period) the formation of peroxides is slow, and this is followed by a period of rapid peroxide formation. The lengths of the induction periods of various oils under similar experimental conditions give an indication of the relative susceptibilities of these oils to become rancid. Orson Bird (1942) has found a relationship between the stability of various liver oils under actual storage conditions and the induction period.

EXPERIMENTAL.

Two c.c. of the sample of shark-liver oil were pipetted into several clean, uniform, flat-bottomed specimen tubes of diameter 2.2 cm. and height 5.0 cm., care being taken to avoid smearing of the oil on the sides of the tubes. They were then set aside in a dust-free chamber or in a constant temperature incubator maintained at the required temperature. At recorded intervals three tubes were taken out and individual determinations made immediately on the peroxide number and vitamin A content, the mean values being taken for graphical representation. The oil used in this experiment had an acid value of 2.52 mg. KOH per g. and a vitamin A content of 4,400 I. U. per g.

The peroxide number was determined essentially as suggested by Wheeler (1932) and expressed in terms of millimols of oxygen per kg. of oil; and vitamin A was estimated by the Carr-Price reaction using the Pulfrich photometer with filter S.61 (Dattatreya Rao, 1944).

The accelerated tests were carried out at room temperature (20°C. to 25°C.), 40°C. \pm 0.5, 50°C. \pm 1, and 100°C. \pm 2 and the results are shown in Graphs 1 to 3 and Tables I to IV.

It was observed that during the test the exposed oils gradually got decolorized and the fishy odour was replaced by a sharp odour.

DISCUSSION.

The rates of formation of peroxides and of destruction of vitamin A at various temperatures were different. The curves for the destruction of the vitamin with time (Graph 2) exhibit the characteristic shape of an autocatalytic reaction more markedly than those for the peroxide number (Graph 1).

TABLE I.

Development of peroxides and destruction of vitamin A at room temperature (20°C. to 25°C.).

Period of storage, hours.	Peroxide number.	Vitamin A *B 1% 1 cm.	Loss in vitamin A, per cent.
0	0	4.84	..
24	3.5
48	6.5
92	12.0
140	18.5
188	23.5	4.71	2.7
240	30.0	4.55	6.0
264	36.0	4.44	8.3
336	67.0	3.29	34.0
480	108.0	2.42	50.0

TABLE III.

Development of peroxides and destruction of vitamin A at 50°C.

Period of storage, hours.	Peroxide number.	Vitamin A *B 1% 1 cm.	Loss in vitamin A, per cent.
0	0	4.84	..
24	10.0	4.68	3.3
48	28.3	3.80	21.5
72	53.5	2.86	41.0
96	66.0	2.47	49.0
144	86.5	1.74	64.0
192	90.0
265	91.0

TABLE II.

Development of peroxides and destruction of vitamin A at 40°C.

Period of storage, hours.	Peroxide number.	Vitamin A *B 1% 1 cm.	Loss in vitamin A, per cent.
0	0	4.84	..
45	10.0
94	26.4	4.33	10.5
117	38.0	3.63	25.0
142	50.5	3.18	34.3
190	77.0	2.59	46.5
237	97.5	2.19	54.7

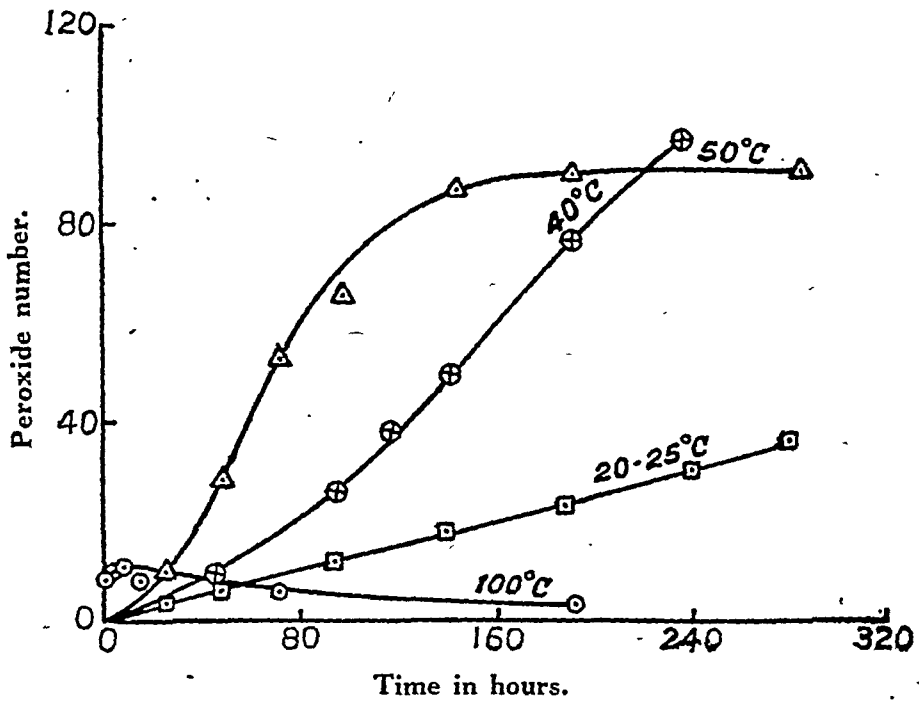
TABLE IV.

Development of peroxides and destruction of vitamin A at 100°C.

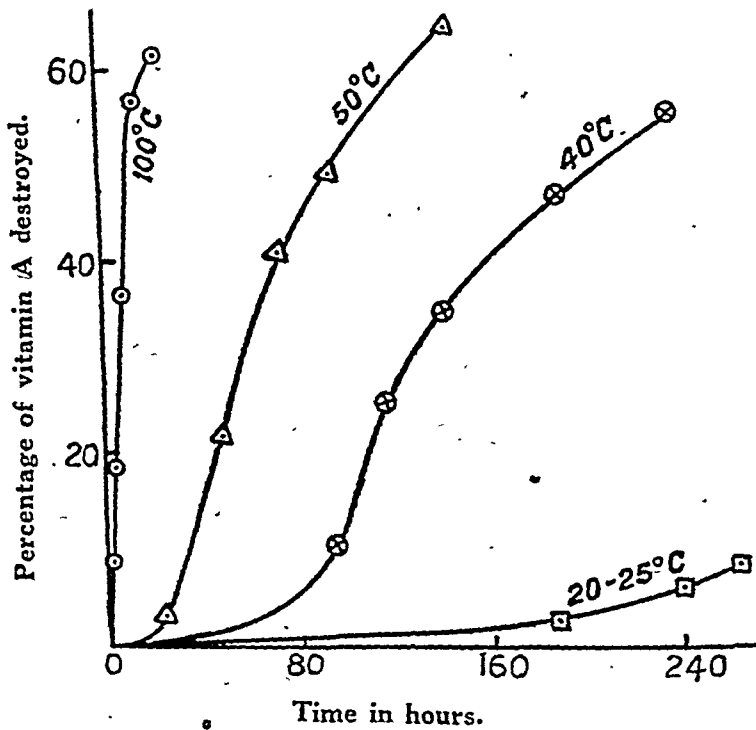
Period of storage, hours.	Peroxide number.	Vitamin A *B 1% 1 cm.	Loss in vitamin A, per cent.
0	0	4.84	..
1	4.9
2	8.5	4.40	8.4
4	10.5	3.96	18.2
8	11.0	3.08	36.4
14	8.3	2.09	56.8
24	8.5	1.76	61.6
72	5.8
192	0.6

*B $\frac{1\%}{1 \text{ cm.}}$ can be converted into I. U. per g. by multiplying with the factor 910.

GRAPH 1.



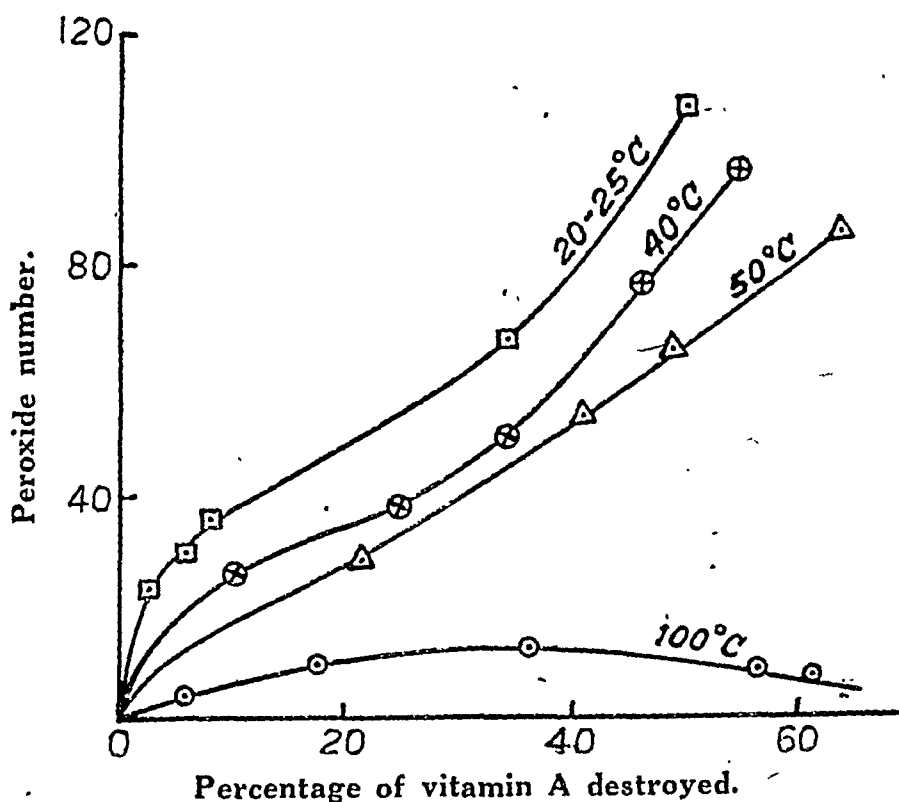
GRAPH 2.



Simons *et al.* (1940) suggest that two reactions are involved in the destruction of vitamin A, viz. first the formation of the peroxides from the unsaturated glycerides and secondly their decomposition with the simultaneous oxidation of the vitamin. It may be seen from Graphs 1 and 2 that the second reaction appeared to be almost as fast as the first at 100°C. so that there was no accumulation of peroxides though the vitamin was destroyed in a very short time. At lower temperatures, however, the peroxide number increased gradually. The destruction of vitamin A was more rapid after the termination of the induction period just as was observed by Lowen *et al.* (*loc. cit.*) in halibut-liver and salmon oils.

Whipple (*loc. cit.*) reported that rancidity produced artificially at 100°C. in cod-liver oil was different from that produced under normal conditions of storage since the vitamin was destroyed at lower peroxide values in the latter. The peroxide number of oils kept in stoppered-bottles does not generally rise to a high figure and the rate of its development is rather erratic since there is no free access to atmospheric oxygen which may explain Whipple's observations. The curves in Graph 3. however, indicate that under the same experimental conditions at different temperatures, the destruction of vitamin A per unit increase in

GRAPH 3.



peroxides was greater at a higher temperature. Peroxides are intermediary compounds in the gradual oxidation of glycerides. They are unstable and the estimated peroxide number gives only the difference between the peroxides formed and those destroyed. The amount destroyed which cannot be measured and which is supposed to be responsible for the oxidation of the vitamin may bear a strict correlation to the amount of vitamin A destroyed.

SUMMARY.

The rates of development of peroxides and of destruction of vitamin A at various temperatures are different. The vitamin is destroyed very rapidly after the termination of the induction period. More of vitamin A is destroyed for the same peroxide number at a higher temperature.

ACKNOWLEDGMENT.

The author thanks Mr. B. N. Banerjee and Professor V. Subrahmanyam for their kind encouragement and keen interest in the work.

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STUDIES ON THE DESTRUCTION OF VITAMIN A IN SHARK-LIVER OIL.

Part IV.

CATALYTIC ACTIVITY OF METALS.

BY

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[Received for publication, August 8, 1944.]

CERTAIN metals are more active than others in hastening the development of rancidity in fats and oils. Emery and Henley (1922) determined the time required for some oils to develop a rancid odour and a positive Kreis reaction in the presence of various metals. They found that the most effective catalyst was copper and the least effective aluminium. Royce (1933) studied the effect of copper on the fading time of methylene blue incorporated in cotton-seed oil. Brocklesby (1941) classified the metals having a pro-oxidative effect on pilchard oil (a semi-drying oil) in the following order of activity: cobalt, manganese, copper, iron, cerium, magnesium, aluminium, zinc and lead; while nickel, tin, cobalt-steels and nickel-chromium-steels had little effect.

King, Roschen and Irwin (1933) studied the influence of various metals both in the dissolved form and in the undissolved form on the induction period of lard aerated at 208°F. The order of activity of the stearates of the metals dissolved in the fat was as follows: copper, manganese, iron, vanadium, chromium, nickel, zinc, aluminium, lead and tin. But the order was different when they were added in the form of sheets of equal area: copper, lead, zinc, tin, iron, aluminium. Both copper stearate at as low a concentration as 0.3 parts per million and the copper sheet independently reduced the induction period to one-sixteenth that of the original lard.

Mattill (1927) observed that the oxidative destruction of vitamins A and E was hastened by the catalytic activity of ferrous iron.

The effect of a few metals commonly used as materials of construction, on the destruction of vitamin A and development of peroxides in shark-liver oil has been studied.

EXPERIMENTAL.

Since it was difficult to obtain some of the metals as sheets, they were added to the oil in the form of a uniform powder, the method of determining the comparative catalytic activity being the same as usual. Filings of different metals were prepared with the same file and only particles of size between 60 and 80 standard mesh were used so that the average particle size of each metal was as uniform as possible. Hence the total surface area of a definite volume of the filings from the different metals may be considered to be practically the same.

The metal filings were taken separately in a 5 c.c. standard flask and packed up to the mark by gently tapping five times and their weights determined. They were later weighed out into a number of specimen tubes (2.2 cm. diameter and 5 cm. height) in the proportion of these weights (Table I). The volume of the powder in each case worked out to be 0.077 c.c. Reilly and Rae (1940) suggest an alternate method where the volume of a definite weight of the powder is determined by displacement of a liquid which is then measured; but this method was not adopted since there is a chance of air bubbles getting entrapped in the powder.

The powders were cleaned by dilute hydrochloric acid, followed by distilled water and dried in vacuum. They were used within 15 minutes after the cleaning to avoid the formation of oxidized films.

TABLE I.

Metal.		Weight of 5 c.c. of the filings, g.	Weight of the filings used for the experiment, g.
Aluminium (Al)	..	6.491	0.1
Zinc (Zn)	..	8.766	0.135
Tin (Sn)	..	10.20	0.157
Mild steel (M.S.)	..	13.31	0.205
Stainless steel (S.S.)	..	15.52	0.239
Nickel (Ni)	..	17.79	0.274
Copper (Cu)	..	19.09	0.291

Two c.c. of shark-liver oil [same sample as the one used in Part III of this series (Dattatreya Rao, 1945, p. 63, this issue)] were pipetted into each of the tubes containing the different metal filings and the tubes were kept in the incubator at 40°C. Periodically three tubes from each lot were taken out and vitamin A content and peroxide number determined individually as before. The mean of the readings are presented in Table II and Graphs 1 to 3:—

TABLE II.

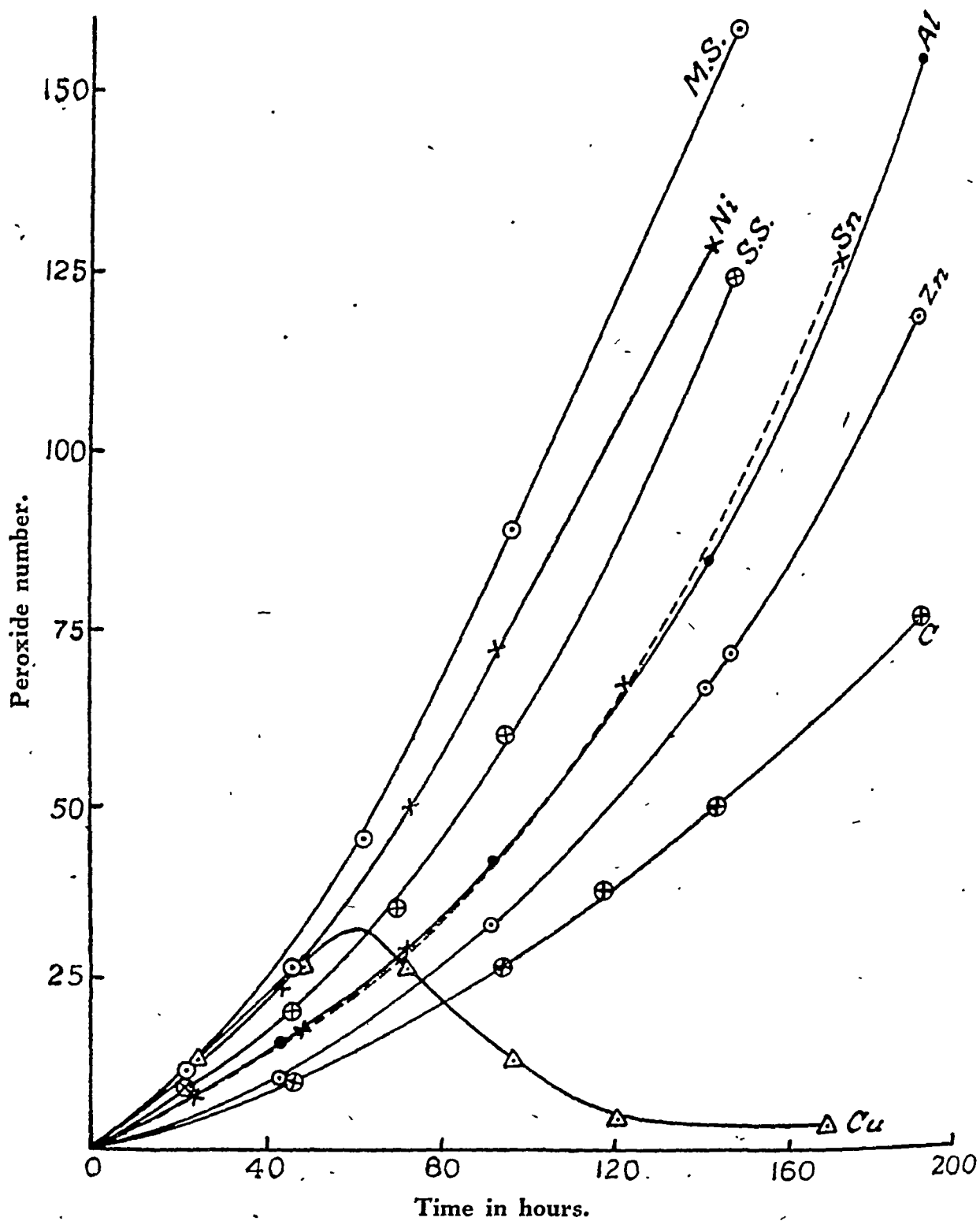
Catalytic influence of metals on the development of peroxides and destruction of vitamin A at 40°C.

Metal.		Period of storage, hours.	Peroxide number.	Loss in vitamin A, per cent.
Zinc C.P.	..	0	0	..
		43	11.0	8.0
		91	32.5	22.5
		139	67.0	38.0
		145	69.5	40.0
		187	120.0	55.0
Aluminium C.P.	..	43	15.8	10.0
		91	41.8	27.5
		139	85.5	46.0
		187	157.0	62.5

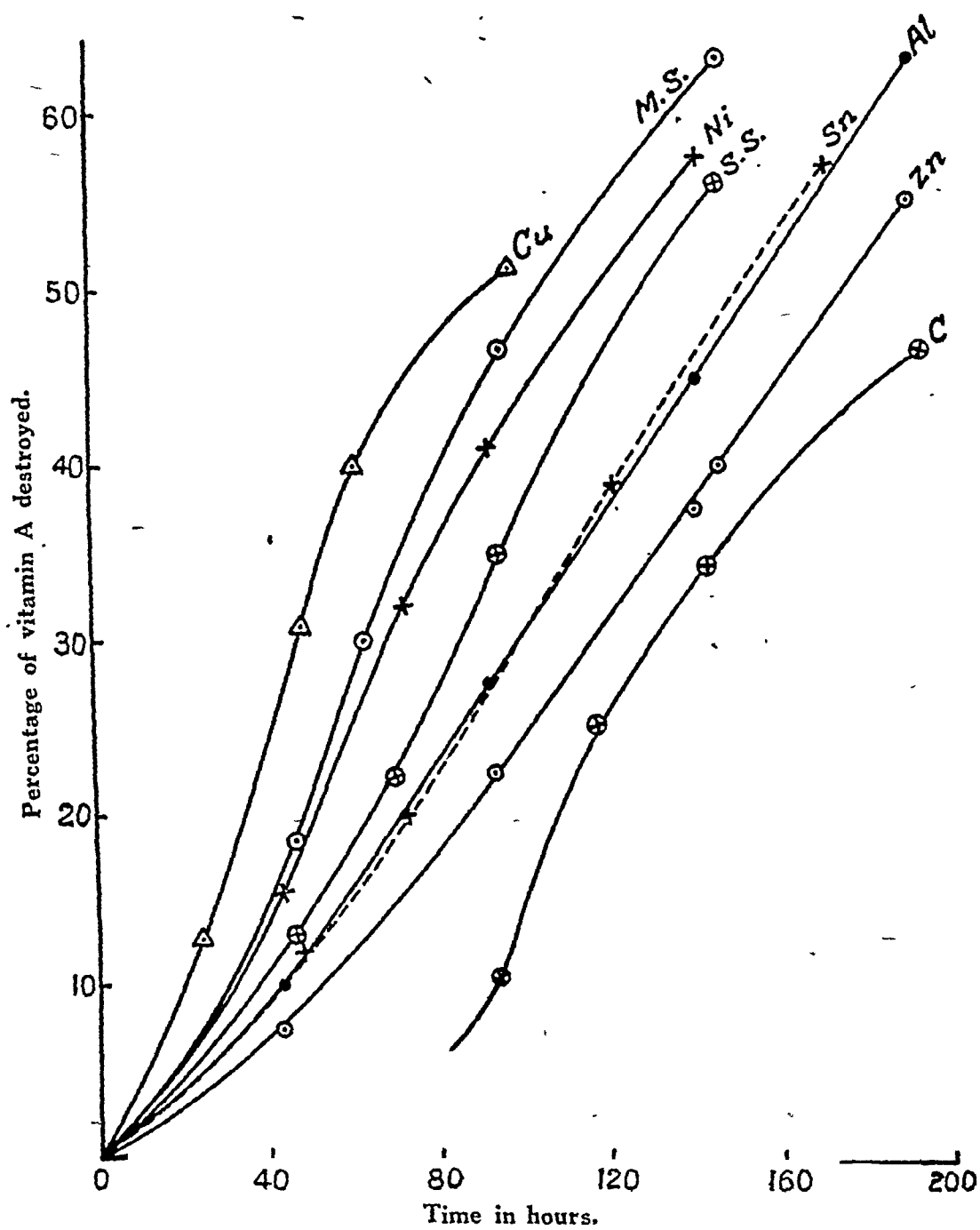
TABLE II—*concl'd.*

Metal.	Period of storage, hours.	Peroxide number.	Loss in vitamin A, per cent.
Tin C.P. 	24	7.0	..
	48	16.9	12.0
	72	29.5	20.0
	120	67.5	39.0
	168	128.0	57.0
Stainless steel (Fe, Cr and Ni)	22	8.5	..
	46	19.5	13.0
	70	35.0	23.0
	94	60.0	36.0
	144	125.0	56.5
Nickel C.P. 	43	23.5	15.5
	72	50.0	32.0
	91	72.2	41.0
	139	130.0	57.5
Mild steel 	22	11.0	..
	46	26.0	18.5
	62	45.0	30.0
	94	89.3	46.5
	144	160.0	63.0
Copper C.P. 	24	12.5	13.0
	48	26.3	31.0
	72	26.0	40.0
	97	12.5	51.3
	121	4.3	..
	169	3.8	..
Control-oil only curve C ..	45	10.0	..
	96	26.4	10.5
	117	38.0	25.0
	142	50.5	34.3
	190	77.0	46.5
	237	97.5	54.7

GRAPH 1.



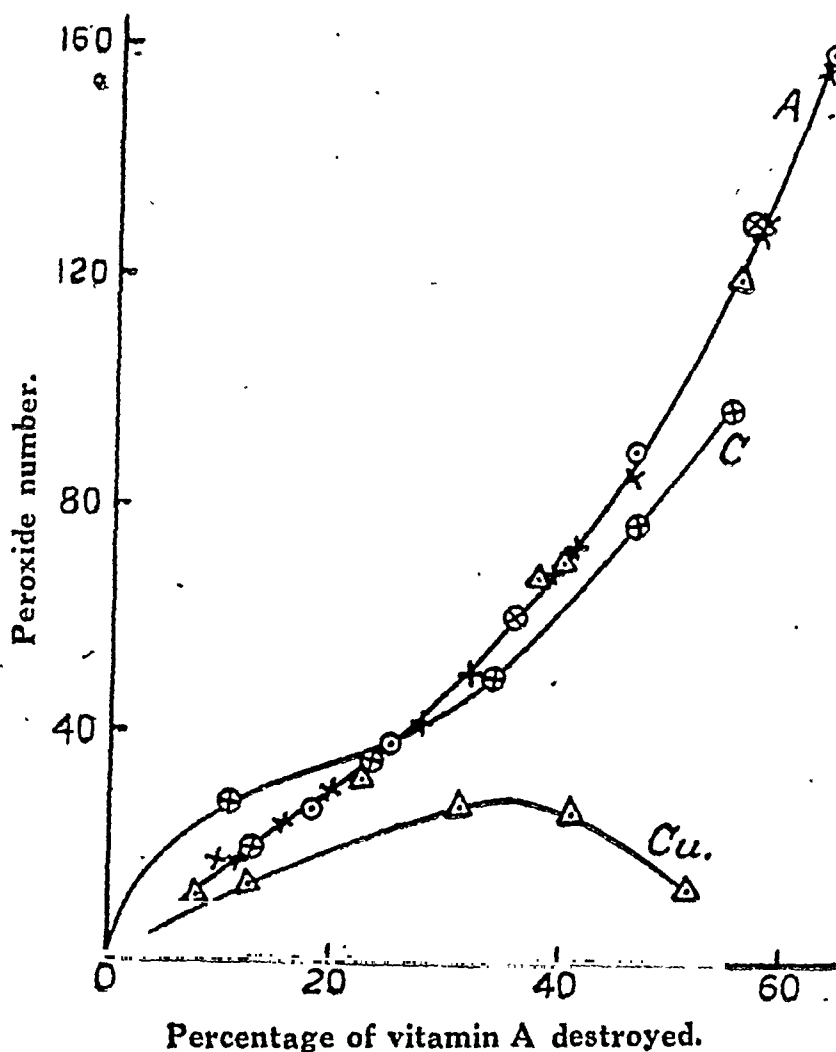
GRAPH 2.



It was observed that after about two days the oil containing the copper became green due to some of the metal dissolving in it. It is possible that the other metals also dissolved in the oil to a certain extent.

It is seen from Graph 2 that the induction period for the destruction of vitamin A was eliminated by all the metals, the rate of oxidation being high right from the start.

GRAPH 3.



DISCUSSION.

All the metals which have been tested were pro-oxidants and they may be classified in the following decreasing order of activity : copper, mild steel (iron), nickel, stainless steel (Ni, Cr and Fe), aluminium and tin, and zinc. Aluminium and tin were almost equally active. The order remains the same with respect to peroxide formation also, the only exception being copper. In the presence of copper the peroxides did not accumulate in shark-liver oil ; nevertheless the destruction of vitamin A was fastest, the condition being similar to the oxidation of the oil at 100°C. (*vide* part III, p. 66, this issue).

It may be seen from curve A, Graph 3, that whatever the metal catalyst may be, the percentage of vitamin A destroyed at any peroxide value is the same since all the points lie on a line. If, as suggested before, two reactions are involved in the destruction of vitamin A, the ratio of the rate of the first reaction (formation of peroxides) to the rate of the second reaction (decomposition of the peroxides with the simultaneous oxidation of the vitamin) appears to be the same with all the metals except copper ; but this ratio is different from that of the original oil.

With copper the second reaction becomes faster than the first after a peroxide number of 25, so that the net peroxide oxygen decreases (Graph 1). This peculiarity of the reaction at 100°C. and in the presence of copper has not been observed with lard and some vegetable oils by other workers (King, Roschen and Irwin, 1933, 1933a).

The order of activity of the metals reported by different authors is not the same. The rate of a catalytic reaction depends on a variety of factors including the presence or absence of promoters, presence or absence of catalytic poisons, surface arrangements in the metals, total surface area, and number and area of the catalytically active patches on the surface. Variation in these factors may result in a different order for the same metals. The nature of the oil also may have some influence on the order. However, there is no difference of opinion regarding the high catalytic activity of copper.

Among the metals studied zinc appears to be the least active and, hence, this metal is to be preferred in the manufacture of equipment for the processing and storage of shark-liver oil. Tin and aluminium are next in the order of preference, the catalytic activity of both being almost equal.

SUMMARY.

All the metals tested had a pro-oxidative effect on shark-liver oil, the order of the metals being: copper, mild steel, nickel, stainless steel, aluminium and tin, and zinc. Aluminium and tin had almost the same activity. At any peroxide number the percentage of vitamin A destroyed is the same with all metals tested excepting copper. In the oil kept at 100°C. there was no accumulation of peroxides in the presence of copper.

The author thanks Mr. B. N. Banerjee and Professor V. Subrahmanyan for their kind encouragement and keen interest in the work.

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THE GROWTH-PROMOTING VALUE OF EGGS.

Part II.

SUPPLEMENTARY VALUE OF EGGS AND PULSES IN A BENGALI DIET.

BY

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AND

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[Received for publication, November 11, 1944.]

MACDONALD and BOSE (1942) demonstrated that the feeding of a typical Bengali village diet to young rats resulted in very unsatisfactory growth and poor health. The addition of eggs to the diet proved very beneficial but still better results were obtained by a further supplement of calcium. A diet consisting of 100 parts Bengali diet *plus* 50 parts eggs, supplemented with 0.5 per cent egg shell gave as good results as a good stock diet. A soya-bean supplement, though beneficial, proved inferior to eggs but a diet consisting of 100 parts Bengali diet *plus* 25 parts eggs *plus* soya was only slightly inferior to the stock diet. Addition of calcium to the soya supplemented diets had no beneficial value. As a diet consisting of 100 parts Bengali diet *plus* 12 parts of egg *plus* soya proved only slightly inferior to diets containing higher proportions of eggs, the authors on economic grounds recommended this diet as suitable for children of seven years. In view of the conflicting literature in regard to the palatability and value of soya-beans in the human diet (Ukil, 1941; Pal, 1939), experiments were carried out to study the supplementary value of eggs and the various common pulses which have long formed part of the human diet in India.

EXPERIMENTAL.

As in the previous work, the basal diet selected was that of the small agriculturist in the district of Dinajpur (Mitra, 1939). The composition of this Bengali diet is given in Table I. The quantities given correspond to the daily intake in ounces per adult man.

TABLE I.

Bengali village diet (daily intake in ounces).

Rice.	Pulses.	Leafy vegetables.	Potatoes.	Brinjals.	Torai.	Mustard oil.	Common salt.	Fish.
25.0	0.4	0.2	3.5	1.75	1.75	0.3	0.1	0.7

The technique followed was similar to that used in the previous investigations (Macdonald and Bose, *loc. cit.*). In each experiment, two male and two female four-week-old rats were fed on the experimental diet for a period of ten weeks. The groups were balanced as regards weight and breeding and the males and females were caged separately. The food mixtures were cooked for half-an-hour on the water-bath in order to duplicate the human diet as closely as possible. Distilled water was given to drink and used for making up the food. To

avoid underfeeding, the amounts fed were regulated so that a small quantity remained in the feeding cups each morning. The composition of the diets used are given in Table II:—

TABLE II.
Composition of the diets.

Number.	Diet.	Composition.
1	B	100 parts Bengali diet.
2	B + egg	63.9 parts Bengali diet plus 36.1 parts egg.
3	B + soya	84.2 parts Bengali diet plus 15.8 parts soya (<i>Glycine Soja Sieb</i>).
4	B + gram	64.2 parts Bengali diet plus 35.8 parts gram (<i>Cicer arictinum</i>).
5	B + green gram	68.4 parts Bengali diet plus 31.6 parts green gram (<i>Phaseolus mungo</i>).
6	B + red lentil	65.1 parts Bengali diet plus 34.9 parts red lentil (<i>Lens esculenta</i>).
7	B + red gram	61.9 parts Bengali diet plus 38.1 parts red gram (<i>Cajanus cajan</i>).
8	B + groundnut (fried)	76.8 parts Bengali diet plus 23.2 parts groundnut (<i>Arachis gigantea</i>).
9	(100B + 12E) + soya	88.2 parts (100 parts Bengali diet plus 12 parts egg) plus 11.8 parts soya.
10	(100B + 12E) + gram	71.7 parts (100B + 12E) plus 28.3 parts gram.
11	(100B + 12E) + green gram	76.6 parts (100B + 12E) plus 23.4 parts green gram.
12	(100B + 12E) + red lentil	73.8 parts (100B + 12E) plus 26.2 parts red lentil.
13	(100B + 12E) + red gram	71.0 parts (100B + 12E) plus 29.0 parts red gram.
14	(100B + 12E) + groundnut	83.3 parts (100B + 12E) plus 16.7 parts groundnut.

The protein levels of the supplemented diets (Nos. 2 to 14) were kept constant at 13.7 to 13.8 per cent of crude protein on dry matter basis. Slonaker (1931) showed that the best growth results in young rats were obtained from a diet with 14 per cent protein. This level of protein also gave the greatest fertility and the longest reproductive span. Table III gives the chemical composition of the various diets.

Numerous workers have shown that too great a discrepancy between the calcium and phosphorus contents leads to growth disturbances. From work on rats, Simmonds (1924) and Kramer and Howland (1932) recommend a Ca : P ratio of 1 : 0.63 and 1 : 0.66 respectively. Workers, however, agree that the permissible range of variation depends on the vitamin D content and that, with optimum amounts of this vitamin, rats can thrive on diets with much wider ratios than those quoted above. Judged on these standards, the Ca : P ratios of all the diets were very wide but they were narrower in the case of soya-supplemented diets than with the other diets. As none of the rations appeared to have adequate amounts of calcium, duplicate diets were made up from each diet by adding 0.5 per cent ground egg shell, so as to raise the calcium contents by about 0.19 per cent. The total number of experimental diets thus amounted to twenty-eight. In the calcium-supplemented diets, the Ca : P ratios ranged from 1 : 0.66 to 1 : 0.86. The rats were protected against vitamin D deficiency by exposing them to direct sunshine for a period of 45 minutes every alternate morning.

On account of seasonal variations on the rate of growth, it is always desirable to carry out all experiments at the same period. Although this was not possible in this extended series of experiments, the results are comparable, as each experiment was designed so as to include a positive control (B + egg + Ca) and a negative control (B). No marked seasonal variation was observed in experiments carried out from October to April.

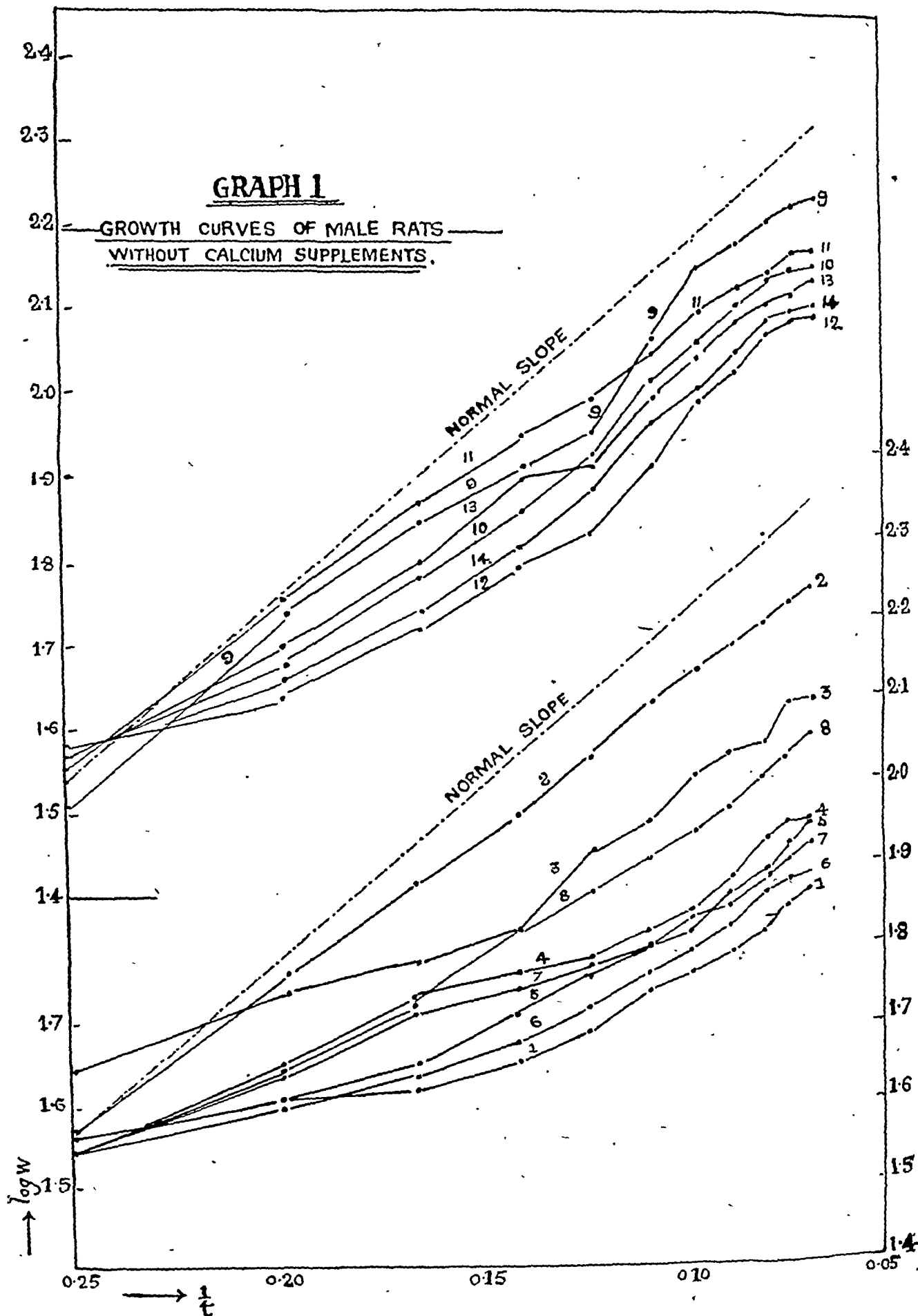
TABLE III.

Percentage composition of the diets.

Number.	Dict.	Moisture.	Crude protein.	Ether extract.	Ash.	Nitrogen-free extract.	Nutritive ratio.	Calories per 100 g.	Ca.	P.	Ca : P.	Crude protein on dry basis.
1	B ..	20.0	6.0	1.0	1.30	71.7	12.3	328	0.036	0.150	1 : 4.2	7.5
2	B + egg ..	40.0	8.3	4.9	1.20	45.6	6.9	266	0.045	0.190	1 : 4.2	13.8
3	B + soya ..	18.5	11.2	3.5	1.85	64.9	6.5	348	0.090	0.240	1 : 2.6	13.7
4	B + gram ..	16.7	11.5	2.6	1.70	67.5	6.4	348	0.050	0.210	1 : 4.2	13.8
5	B + green gram ..	17.0	11.5	1.6	1.91	68.0	6.2	340	0.046	0.225	1 : 4.9	13.8
6	B + red lentil ..	17.4	11.4	1.4	1.50	68.3	6.3	339	0.046	0.226	1 : 4.9	13.8
7	B + red gram ..	16.1	11.6	1.0	2.10	69.2	6.2	339	0.057	0.223	1 : 3.9	13.8
8	B + groundnut ..	15.8	11.5	11.4	1.50	59.8	7.4	398	0.036	0.198	1 : 5.5	13.7
9	(100B + 12E) + soya	22.5	10.7	3.7	1.68	61.4	6.5	330	0.080	0.230	1 : 2.9	13.8
10	(100B + 12E) + gram	21.9	10.8	2.9	1.60	62.8	6.4	329	0.048	0.200	1 : 4.2	13.8
11	(100B + 12E) + green gram	22.2	10.8	2.2	1.80	63.0	6.3	322	0.045	0.213	1 : 4.7	13.9
12	(100B + 12E) + red lentil ..	22.3	10.7	2.0	1.40	63.6	6.4	322	0.044	0.215	1 : 4.9	13.8
13	(100B + 12E) + red gram ..	21.2	10.9	1.7	1.10	65.1	6.3	326	0.053	0.212	1 : 4.0	13.8
14	(100B + 12E) + groundnut ..	21.8	10.7	9.3	1.40	56.8	7.3	363	0.038	0.195	1 : 5.1	13.7

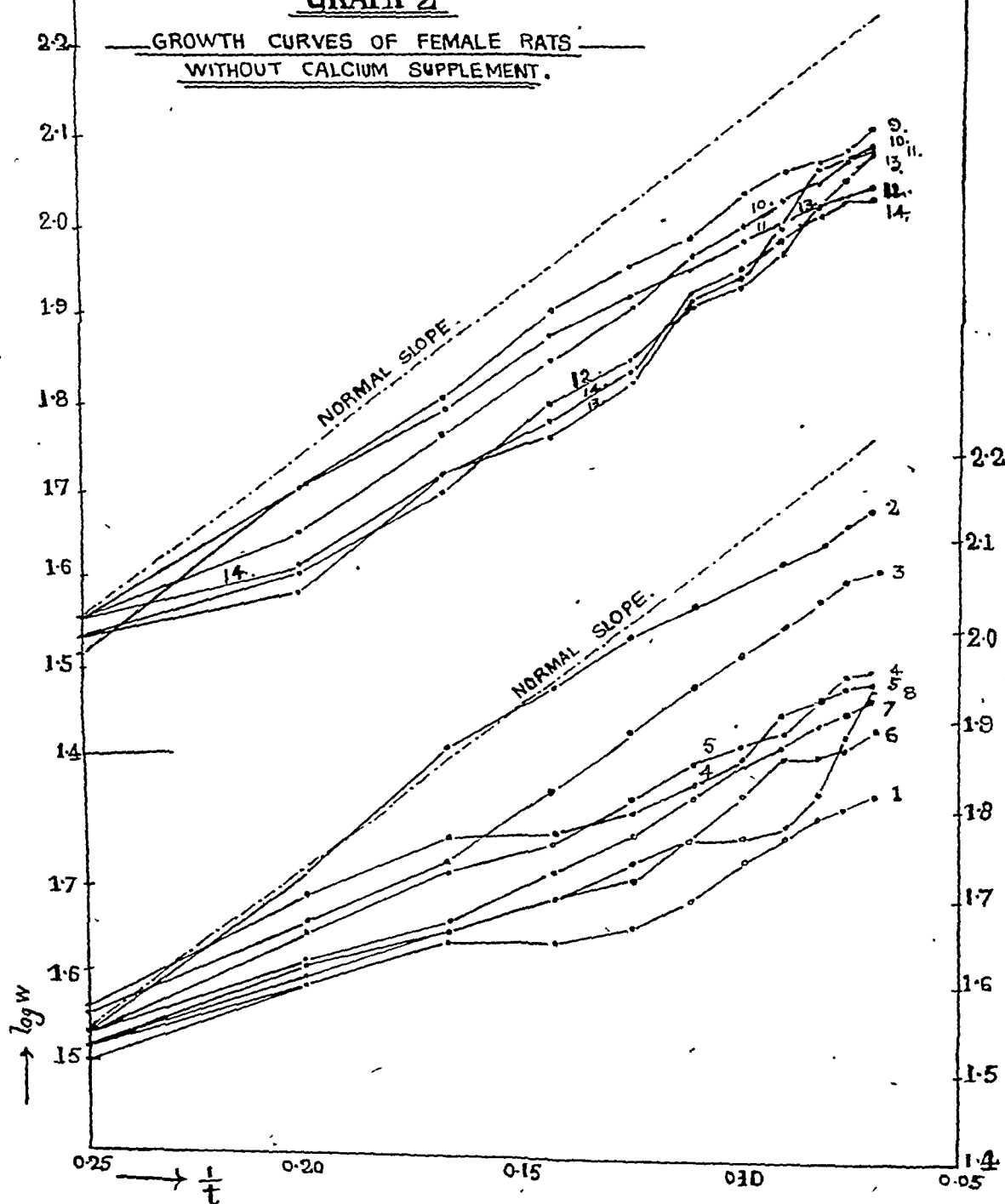
GRAPH 1

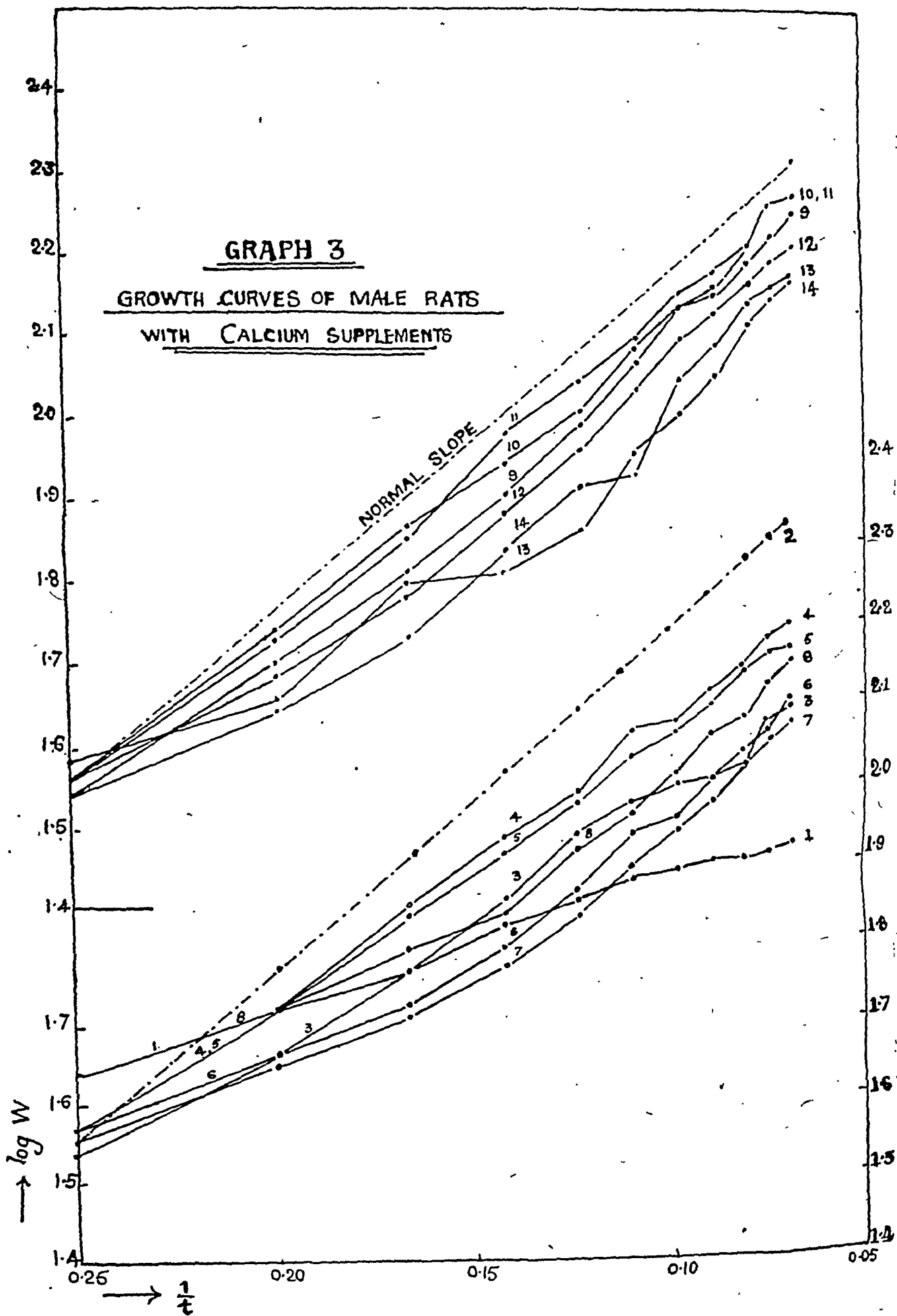
GROWTH CURVES OF MALE RATS
WITHOUT CALCIUM SUPPLEMENTS.



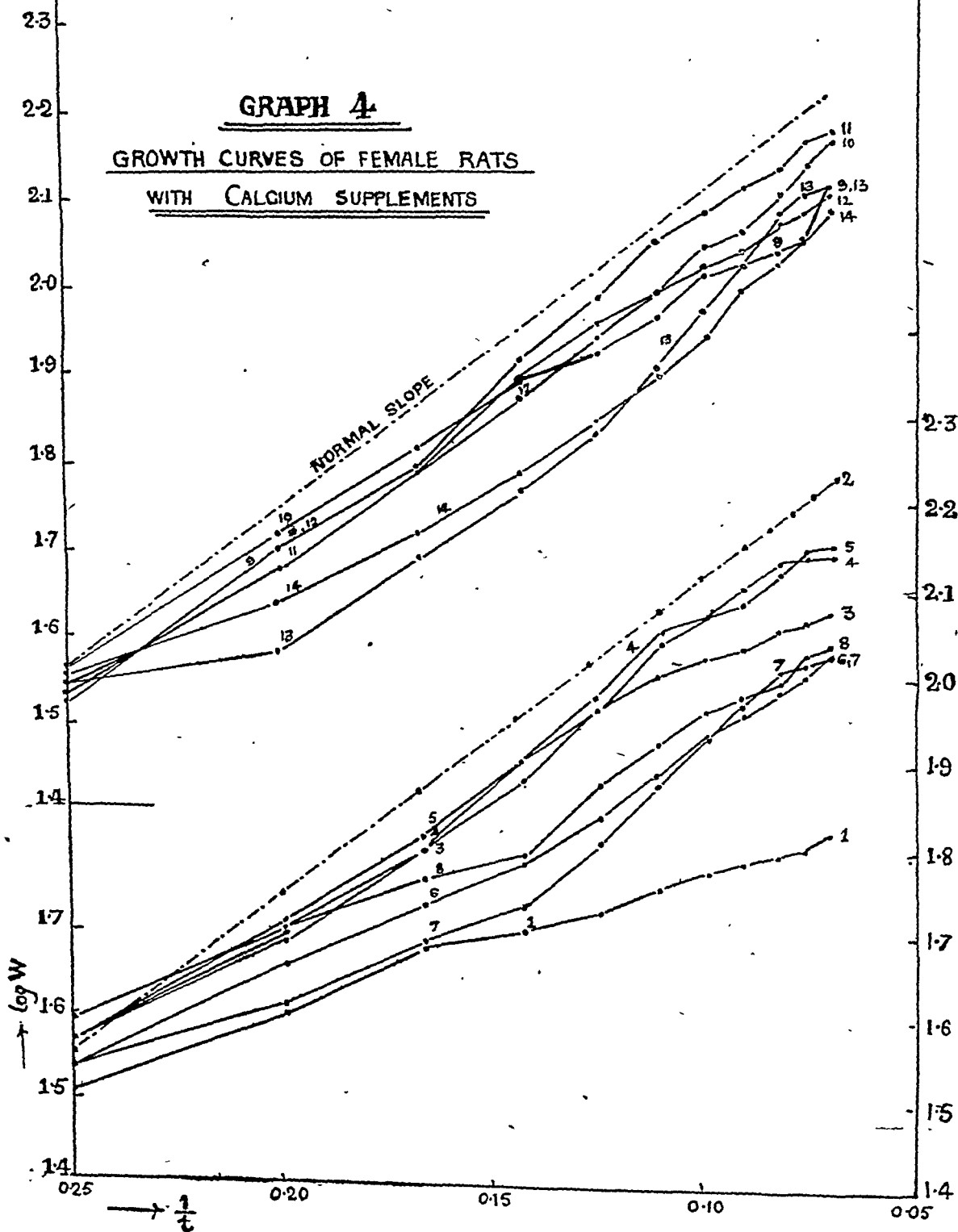
GRAPH 2

GROWTH CURVES OF FEMALE RATS
WITHOUT CALCIUM SUPPLEMENT.





GRAPH 4
GROWTH CURVES OF FEMALE RATS
WITH CALCIUM SUPPLEMENTS



GROWTH RESULTS.

Zucker, Hall, Young and Zucker (1941) and Zucker and Zucker (1943), from experiments on rats on good stock diets, observed that the growth curve could be put into a straight line form if the logarithm of the weight is plotted against the reciprocal of time in any appropriate units. They found that dietary deficiencies resulted in sub-optimal growth curves, the shapes of which were characteristic of the deficiency concerned. For instance, with a moderate protein deficiency, the curvilinear log-reciprocal plot tended to approach the optimum growth straight line at later stages of growth. This is attributable to decreasing protein requirements in older animals. On the other hand, dietary requirements such as thiamine, which increase with age, caused an increasing departure from growth normal as time went on. Differences of genetic origin had no effect on the shape of the growth curve and means of different body sizes could be plotted as parallel straight lines.

In this investigation, the growth results have been recorded as the log-reciprocal plots in order to facilitate interpretation of the data and for comparison of the results. As the result obtained in each experiment were very similar for each of the diets, the combined average growth results of the male and female rats on each diet are given in Graphs 1, 2, 3 and 4. In order to avoid over-crowding, the curves in each graph have been separated into two groups, each of which is plotted with a separate weight scale and origin. The average weekly gains in weights in grammes made by the rats on the different diets during the ten weeks' experimental period are given in Table IV:—

TABLE IV.
Average weekly gains in weight in g.

Number.	Diet.	MALE.		FEMALE.	
		No calcium supplement.	With calcium supplement.	No calcium supplement.	With calcium supplement.
1	B	3.6	4.0	3.3	3.4
2	B + egg	13.1	17.2	9.9	13.1
3	B + soya	8.9	8.8	7.9	8.0
4	B + gram	5.4	11.7	5.3	10.2
5	B + green gram	5.3	10.9	5.0	10.3
6	B + red lentil	4.3	8.8	4.1	6.9
7	B + red gram	4.7	8.1	4.8	7.0
8	B + groundnut	6.8	9.6	5.1	6.8
9	(100B + 12E) + soya	13.9	13.8	9.7	9.8
10	(100B + 12E) + gram	10.8	14.8	8.6	11.2
11	(100B + 12E) + green gram	11.1	14.6	8.6	11.6
12	(100B + 12E) + red lentil	8.7	12.2	7.5	9.5
13	(100B + 12E) + red gram	10.0	10.6	8.6	9.8
14	(100B + 12E) + groundnut	9.1	11.0	7.2	8.6

The figures in Table V have been calculated on the basis of Aykroyd's (1938) Indian standards for school children aged 6 to 9 years. The weight in grammes and the calorific value of each diet necessary to give a protein intake of 60 grammes per day has been calculated.

The number of eggs with internal contents weighing 45 grammes and the weights of the various supplements required are also given for each of the diets.

TABLE V.

Calculated diets to give a daily protein intake of 60 grammes.

Diet.	Consumption in g.	Calories.	Number of eggs required.	Pulses required in g.
B	1,000	3,280
B + egg	723	1,923	5.8	..
B + soya	536	1,865	..	85
B + gram	522	1,817	..	187
B + green gram	522	1,775	..	165
B + red lentil	526	1,783	..	184
B + red gram	517	1,753	..	197
B + groundnut	522	2,078	..	121
(100B + 12E) + soya	561	1,851	1.18	66
(100B + 12E) + gram	556	1,829	0.95	157
(100B + 12E) + green gram	556	1,790	1.01	130
(100B + 12E) + red lentil	561	1,806	0.99	147
(100B + 12E) + red gram	550	1,793	0.93	160
(100B + 12E) + groundnut	561	2,036	1.11	94

DISCUSSION.

It will be seen from the graphs that the rats fed on the Bengali diet grew very unsatisfactorily and the logarithms of their weights when plotted against the reciprocals of time showed an ever-increasing departure from the normal straight line. The addition of calcium as ground egg shell though it improved the Ca : P ratio had very little beneficial effect as the diet still remained poor in protein and vitamins, all of which are limiting factors for growth.

With eggs as the sole supplement to the Bengali diet, the best results were obtained with a diet containing 63.9 parts Bengali diet *plus* 36.1 parts eggs. The log-reciprocal plot of the results on this diet only showed a slight departure from the normal line. The addition of calcium in the form of egg shell improved the (B + egg) diet and the log-reciprocal plot of the growth results gave almost a straight line indicating a well-balanced diet.

Supplements of soya-beans, gram, green gram, red lentil, red gram and groundnut to the Bengali diet proved beneficial as regards growth but they all proved markedly inferior to the egg supplemented Bengali diet. The nutritive value of the supplements stood in the following descending order of merit—soya-beans, groundnut, gram, green gram, red gram and red lentil. No benefit was obtained by adding calcium to the (B + soya) diet, but calcium proved beneficial when fed with all the other supplemented Bengali diets. In these groups gram and green gram gave the best results, soya-beans and groundnut were appreciably lower in value, while red lentil and red gram gave the poorest results.

Supplements of soya-beans, gram, green gram, red lentil, red gram and groundnut proved much more effective with the (100B + 12E) diet than with the Bengali diet only. The nutritive values of these vegetable protein-rich supplements when added to the (100B + 12E) diet ranged themselves in the following descending order—soya-beans, green gram, gram, red gram, groundnut and red lentil. All the rations, except the soya one, were improved by the addition of calcium in the form of egg shell. With calcium supplements, the (100B + 12E) diet supplemented with green gram and gram gave even better results

than the (B + egg) diet but slightly inferior results than the (B + egg) diet supplemented with calcium. The (100B + 12E) diet supplemented with soya also gave slightly better results than the (B + egg) diet. Red lentil, red gram and groundnut as supplements to the (100B + 12E) + Ca diet in order of merit, proved somewhat inferior to the (B + egg) diet.

When no calcium was added to the diet, the soya-bean supplement proved definitely superior to the other vegetable protein supplements either as the sole protein rich supplement to the Bengali diet or in combination with the Bengali diet and eggs. However, when calcium was added, supplements of green gram and gram both proved superior to the soya-bean supplement. The necessity of including calcium along with diets containing considerable amounts of vegetable proteins is of very considerable practical importance from the human nutritional aspect for, in many areas, the amount of calcium in the diet must be somewhat deficient owing to low consumption of calcium-rich foods, such as milk and greens. It would appear that one of the advantages in using soya is that it is comparatively rich in calcium but if this necessary element is made good through some other source even better results are obtained with green gram and gram.

The Bengali diet alone is very unsatisfactory from the nutritional aspect for, in order to get the requisite amounts of 60 grammes protein for children aged 6 to 9 years, the total amount of food consumed and its calorific value are much in excess of requirements. The Bengali diet + egg, especially when supplemented with calcium, gave excellent growth results but this type of diet is impractical as the optimum supplement of 5.8 eggs (internal content 45 g.) per child is beyond the means of the average family income.

From the economic and nutritional aspect, the Bengali diet *plus* an egg a day *plus* the requisite amounts of pulses can therefore be recommended as satisfactory for growing children.

SUMMARY.

1. A typical Bengali village diet gave very poor growth results, when fed to rats.
2. Eggs as the sole protein supplement to a Bengali diet gave good growth results but even better results were obtained with eggs *plus* calcium.
3. Supplements of soya-beans, gram, green gram, red lentil, red gram and groundnut to the Bengali diet, all improved the growth rate but proved inferior to eggs.
4. The addition of calcium in the form of egg shell to the various vegetable protein supplemented diets had a beneficial effect on all the diets except that containing soya-beans. With a calcium supplement, green gram and gram proved superior to soya-beans. Green gram, gram and soya all proved superior to red gram, groundnut and red lentil supplements.
5. In the absence of a calcium supplement, soya-beans as the sole protein supplement or in combination with egg proved superior to the other vegetable protein supplements.
6. Better results were obtained with all the vegetable protein supplements in combination with eggs than when fed alone.
7. From the purely economic aspect of human nutrition good results should be obtained with children from 6 to 9 years on a Bengali diet supplemented with one egg a day and the necessary amount of pulses to bring the protein level of the diets to 60 grammes per day.

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BIOLOGICAL VALUE OF PROTEINS FROM MUSCLE MEAT OF COW, BUFFALO AND GOAT.

BY

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INTRODUCTION.

WITH the meat-eating section of the population goat-meat is a very popular article of diet in Bihar. The consumption of buffalo-meat is usually confined to the poorer section of the Mohammedan community, whereas beef is taboo to the Hindus but is consumed by the Mohammedans, Christians and aboriginals. The biological value of beef muscle has been determined in other countries but that of goat or buffalo-meat has not so far been assessed. In the present investigation an attempt has been made to estimate the biological value of the proteins of muscle meats of cow (beef), buffalo and goat by the balance-sheet method.

EXPERIMENTAL.

All the three kinds of meat were purchased from local butchers and the muscle meat was separated from the bones and cleaned of the fasciæ as far as practicable. Enough of each sample was purchased to last throughout the experiment. The cleaned sample of muscle was dried over a water-bath, powdered and stocked in big glass-stoppered bottles and stored in the refrigerator for use. After the experiment with one type of meat was finished the next type was purchased and similarly treated and stored. The meat powders were never stored for more than a week before the feeding experiment was actually started.

On analysis by chemical methods 100 g. of each of the three samples of meat powder were found to contain the following :—

	Cow, g.	Buffalo, g.	Goat, g.
Moisture	4.83	4.50	2.60
Nitrogen	13.03	12.96	11.37
Ether extractives	5.85	4.49	19.82
Mineral matter	4.25	5.20	4.20
Carbohydrates by difference ..	3.58	4.86	2.33

The comparatively low percentage of ether extractives or fat noticed in the meat powders prepared from beef and buffalo-meat confirms the local belief about the ill-nourished condition of the cattle slaughtered.

The biological value of proteins present in each of the meat powders was determined by the balance-sheet method using laboratory-bred adult white rats as experimental animals. The composition of the nitrogen-free diet as also the details of the technique followed have been fully described in previous communications from these Laboratories (Mitra and Mittra,

1942, 1943). The animals were fed at 10 and 15 per cent levels of protein intake and the details of the test diets used are given in Table I:—

TABLE I.
Composition of test diets in grammes.
(Calorific value about 2,500.)

Items of food.	Cow.		BUFFALO.		GOAT.	
	10 per cent level.	15 per cent level.	10 per cent level.	15 per cent level.	10 per cent level.	15 per cent level.
Meat powder	74.8	112.2	75.3	113.0	86.0	129.0
Corn starch	279.0	277.0	278.0	276.0	277.0	275.0
Sugar cubes	54.0	54.0	54.0	54.0	54.0	54.0
Coco-nut oil	89.3	73.6	90.7	75.7	77.0	55.2
Salt mixture	24.0	24.0	24.0	24.0	24.0	24.0
Calcium carbonate ..	6.0	6.0	6.0	6.0	6.0	6.0

The average intake and output of nitrogen per rat on each of the test diets is given in Table II:—

TABLE II.
Average intake and output of nitrogen per rat per day in grammes.
10 per cent level.

Cow.

Rat unit number.	NITROGEN-FREE DIET.			EXPERIMENTAL OR TEST DIET.				Relative digestibility co-efficient.	Relative biological value.
	Dry food intake, g.	NITROGEN EXCRETED, g.		Dry food intake, g.	Total nitrogen intake, g.	NITROGEN EXCRETED, g.			
		Fæces.	Urine.			Fæces.	Urine.		
1 C ..	11.4	0.0264	0.0290	9.4	0.1828	0.0270	0.0880	99.7	67.6
2 C ..	11.7	0.0225	0.0291	11.7	0.2258	0.0312	0.0910	96.2	71.5
3 C ..	13.1	0.0316	0.0309	12.5	0.2379	0.0395	0.1125	96.7	64.5
8 C ..	10.5	0.0287	0.0268	8.3	0.1624	0.0299	0.0779	99.3	68.3
9 C ..	12.4	0.0314	0.0259	11.8	0.2104	0.0447	0.0916	93.7	66.7
10 C ..	12.6	0.0300	0.0385	10.4	0.1792	0.0327	0.0866	98.0	72.8

10 per cent level.

Buffalo.

1 B ..	15.4	0.0475	0.0418	16.5	0.3101	0.0478	0.1664	99.9	59.8
2 B ..	15.4	0.0377	0.0420	16.5	0.3100	0.0562	0.1574	94.0	60.4
3 B ..	15.1	0.0362	0.0347	15.3	0.2874	0.0581	0.1545	92.4	54.9
8 B ..	15.4	0.0373	0.0358	16.4	0.3079	0.0602	0.1582	92.6	57.1
9 B ..	11.9	0.0331	0.0498	13.5	0.2544	0.0475	0.1338	94.3	65.0
10 B ..	14.8	0.0340	0.0299	12.9	0.2436	0.0469	0.1234	94.7	59.5

TABLE II—concl'd..

10. per cent level.

Goat.

Rat unit number.	NITROGEN-FREE DIET.			EXPERIMENTAL OR TEST DIET.				Relative digestibility co-efficient.	Relative biological value.
	Dry food intake, g.	NITROGEN EXCRETED, g.		Dry food intake, g.	Total nitrogen intake, g.	NITROGEN EXCRETED, g.			
		Fæces.	Urine.			Fæces.	Urine.		
1 G ..	12.9	0.0259	0.0381	15.1	0.2869	0.0474	0.1392	92.2	61.9
2 G ..	13.8	0.0313	0.0302	15.2	0.2890	0.0434	0.1422	95.9	59.6
3 G ..	8.7	0.0211	0.0374	14.1	0.2652	0.0463	0.1365	90.5	58.7
8 G ..	11.6	0.0297	0.0236	15.2	0.2879	0.0427	0.1312	95.8	60.9
9 G ..	14.9	0.0379	0.0510	15.0	0.2831	0.0397	0.1472	99.3	65.8
10 G ..	15.0	0.0315	0.0481	12.5	0.2385	0.0379	0.1514	97.2	55.5

15 per cent level.

Cow.

4 C ..	12.9	0.0248	0.0250	12.9	0.3467	0.0299	0.1406	98.5	64.2
5 C ..	12.2	0.0289	0.0290	12.2	0.3271	0.0337	0.1683	98.5	56.8
6 C ..	11.5	0.0255	0.0289	11.5	0.3042	0.0296	0.1464	98.7	60.9
7 C ..	10.4	0.0215	0.0359	11.4	0.3047	0.0310	0.1770	96.9	52.2
11 C ..	13.3	0.0302	0.0391	9.9	0.2610	0.0320	0.1888	99.3	42.3
12 C ..	14.1	0.0283	0.0330	9.7	0.2563	0.0318	0.1703	98.6	45.7

15 per cent level.

Buffalo.

4 B ..	14.9	0.0406	0.0355	13.7	0.3623	0.0430	0.2158	99.3	49.9
5 B ..	15.0	0.0369	0.0341	16.3	0.4386	0.0466	0.2705	97.8	44.9
6 B ..	14.8	0.0345	0.0325	13.7	0.3613	0.0430	0.2175	97.7	47.3
7 B ..	15.4	0.0370	0.0366	16.1	0.4340	0.0534	0.2407	96.2	51.2
11 B ..	12.8	0.0329	0.0352	12.5	0.3275	0.0518	0.2104	94.2	43.2
12 B ..	14.4	0.0334	0.0311	14.7	0.3939	0.0449	0.2525	97.1	42.1

15 per cent level.

Goat.

4 G ..	11.5	0.0169	0.0358	11.2	0.3019	0.0377	0.1852	93.1	46.9
5 G ..	13.6	0.0256	0.0324	12.8	0.3492	0.0432	0.1987	95.0	49.8
6 G ..	9.6	0.0215	0.0269	12.1	0.3296	0.0477	0.1944	92.1	44.8
7 G ..	13.3	0.0298	0.0406	14.7	0.4016	0.0561	0.2392	93.5	47.1
11 G ..	14.6	0.0302	0.0376	14.3	0.3929	0.0482	0.2606	95.4	40.5
12 G ..	14.9	0.0328	0.0536	15.6	0.4276	0.0479	0.2564	96.6	50.8

RESULT.

Of the three kinds of muscle meat tested the average biological value of beef was found to be the highest, e.g. 68·6 at a 10 per cent level and 53·7 at a 15 per cent level of protein intake. Mitchell and Carman (1926) had obtained an average value of 69 at 9·4 per cent level with beef muscle dried, powdered and ether extracted. The individual figures were subjected to 't' test and it was found that at 10 per cent level the biological value of beef was *significantly* superior to that of either buffalo-meat or goat-meat.

Kind of muscle meat.			RELATIVE DIGESTIBILITY CO-EFFICIENT.		RELATIVE BIOLOGICAL VALUE.	
			10 per cent level.	15 per cent level.	10 per cent level.	15 per cent level.
Cow	97·3	98·4	68·6	53·7
Buffalo	94·7	97·1	59·5	46·4
Goat	95·2	94·2	60·4	46·7

At 15 per cent level of intake, however, the superiority noticed in favour of beef was not statistically *significant* as P was found to lie between 0·1 and 0·2. Such a conclusion is not at all surprising in view of the fact that a good deal of variation was noticed within the group itself in the case of beef at 15 per cent level. The slight superiority noticed in the case of goat-meat over buffalo-meat at both the levels of intake was not significant. In all the three kinds of meat, however, the 10 per cent level of intake was found to be *significantly* superior to that at 15 per cent level.

SUMMARY.

The average biological value of the protein of muscle meat from cow, buffalo and goat were estimated by the balance-sheet method at 10 and 15 per cent levels of protein intake. Ten per cent level of intake was found to be superior to that at 15 per cent. No significant difference could be found between the figures for buffalo-meat or goat-meat, whereas at 10 per cent level beef was found to be significantly superior to buffalo-meat or goat-meat.

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THE FOOD VALUE OF A FURTHER BATCH OF EDIBLES.

ESTIMATED BY CHEMICAL METHODS.

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INTRODUCTION.

IN a series of communications from these Laboratories (Mitra, 1938; Mitra and Mittra, 1941, 1942, 1943) the percentage composition of proteins, fats, ash, moisture, carbohydrates, calcium and phosphorus of 250 different kinds of food has already been published. The present study, which forms the final report of the series, deals with the food value of 40 kinds of edibles not analysed so far. In the series some of the more uncommon edibles, such as frog and rat meat, have been included as these analyses had to be undertaken in connection with dietary survey operations in Bihar during which these foods were found in the list of edibles actually consumed by groups of population under investigation.

EXPERIMENTAL.

The procedure reported in previous communications (*loc. cit.*) was followed: A good deal of difficulty was experienced in obtaining samples of mushrooms in fresh condition as the specimens often withered during transit from rural markets situated at long distances from these Laboratories and for each sample five or six attempts had to be made. The results of analysis are given in the Table below.

DISCUSSION.

Tanguni ka chawal is looked down upon as a very inferior millet but the results of analysis show that it is a superior article of diet as compared to rice. *Bhat jhinga* is the name given to immature shrimps resembling cooked rice grains. The calcium content has been found to be very high as it is eaten with the soft chitinous jacket and has been analysed in that state. From the results of experiment carried out recently on the availability of calcium from bones of small fish by Basu one feels justified in assuming that the chitinous jacket of *bhat jhinga* is a rich source of calcium for the human organism. *Boordood* or white ants are eaten either baked or fried in oil in certain parts of Chota Nagpur. The Indian bull frog and the field rat are consumed by the lower-class people. In this connection it may be pointed out that frog is considered a delicacy in certain parts of civilized world and Furnas and Furnas (1937) have cited an instance when students of a famous university experimented with rat meat and found that for really hungry persons it was a good dish. The meat of common mussel which is eaten by poorer-class people has a high calcium content as in the case of snail's meat.

People who are fond of mushrooms often sing the praise of this edible in no uncertain terms. There is a popular belief that mushroom curry tastes like meat curry but the protein figures in all the 9 varieties analysed do not justify the idea that mushrooms are a meat substitute. The flour made from the kernel of mango seeds was found to be consumed by a section of the people during the food crisis last year. The sample analysed was procured from one such consumer and was said to have been processed or subjected to thorough washing to get rid of its astringent taste after the kernel was dried and ground. The pressed ground-nut cake was found to possess a very high protein percentage and if popularized as human food is likely to improve poor rice eater's diet. The fruits of sal tree and the roasted kernel

TABLE.

Food values of edible portions in grammes per cent.

Serial number.	Hindi or local name.	English name.	Latin name.	Moisture, g.	Protein, g.	Other extractives, g.	Carbohydrates, g.	Mineral matter, g.	Calcium, g.	Phosphorus, g.	Crude fibre, g.
GRAIN FOODS.—											
1	Barai dal	<i>Phaseolus mungo</i> var.	6.50	21.58	0.85	63.30	3.50	0.109	0.458	4.27
2	Tangani ka chawal	<i>Setaria italica Beauv</i>	9.75	10.22	3.25	75.80	0.98	0.029	0.165	..
FLESH FOODS.—											
3	Bhat jhinga ..	Immature shrimps	<i>Palæmon</i> sp.	75.49	14.83	2.79	3.29	3.60	1.201	0.300	..
4	Boordood ..	Winged white ants	2.09	49.28	44.49	..	4.14	0.048	0.457	..
5	Dhauns ..	Indian bull frog	<i>Rana tigrina</i>	79.06	19.58	0.40	..	0.96	0.010	0.176	..
6	Harna chuha ka gost ..	Field rat's meat	73.90	23.62	1.04	..	1.44	0.030	0.242	..
7	Katchua ka anda ..	Turtle's egg	76.01	12.19	6.70	3.65	1.45	0.093	0.299	..
8	Situwa ..	Fresh water mussel	<i>Lamellidens marginalis</i>	79.45	14.50	1.61	2.13	2.31	0.592	0.406	..
9	Soega machli ..	Fish	<i>Eutropichthys vacha</i>	75.75	20.83	2.32	..	1.10	0.023	0.199	..
FRUITS.—											
10	Borooee	<i>Gardenia gummiifera</i>	74.06	2.04	0.28	17.57	1.32	0.068	0.064	4.73
11	Jamiri nimboo ..	Lime	92.87	0.60	0.31	5.67	0.34	0.027	0.016	0.21
12	Jilebi (biletti imli)	<i>Pithecolobium dulce</i>	76.66	3.76	0.15	17.98	0.66	0.009	0.048	0.89
13	Jurmata	<i>Canthium didymum</i>	46.04	4.80	0.25	43.28	2.21	0.115	0.101	3.42
14	Tirkol ka phal	93.88	0.74	0.13	4.26	0.99	0.034	0.046	...

MUSHROOMS.—

15	Bali or lava chhattoo ..	Edible mushroom	87.68	5.24	0.90	4.30	1.88	0.009	0.170	..
16	Bhorra chhattoo	94.37	1.86	0.85	2.16	0.76	0.004	0.103	..
17	Chhotā karhani chhattoo or lagra chhattoo.	87.97	6.31	0.92	3.03	1.77	0.007	0.155	..
18	Dingil chhattoo or baskukhri chhattoo.	85.50	6.35	1.02	4.05	3.08	0.003	0.186	..
19	Patra chhattoo ..	Edible mushroom	87.36	3.05	0.67	8.16	0.76	0.003	0.044	..
20	Phutka chhattoo (rugroo) ..	Puff-ball mushroom	80.91	3.67	0.40	14.12	0.90	0.016	0.073	..
21	Pual chhattoo ..	Straw mushroom	91.24	5.84	0.42	1.32	1.18	0.002	0.115	..
22	Tila chhattoo ..	Ant-hill mushroom	85.88	7.24	1.30	3.37	2.21	0.004	0.174	..
23	Tumba chhattoo ..	Gourd mushroom	90.00	2.31	0.89	5.92	0.88	0.003	0.056	..

Collybia sp.

Lycoperdon sp.

Volvariā terastius

Entoloma macrocarpum

Bovisia gigantea

MISCELLANEOUS.—

24	Am ka guthli ka atte ..	Flour from pulp of mango seed.	<i>Mangifera indica</i>	6.14	6.20	11.34	68.99	4.30	0.411	0.052	3.03
25	Deshi chini (ukh ka) ..	Cane sugar (home made)	<i>Saccharum officinarum</i>	1.15	0.24	0.02	98.23	0.36	0.028	0.004	..
26	Chini ..	Cane sugar (commercial quality).	<i>Saccharum officinarum</i>	0.42	0.14	0.01	99.38	0.05	0.012	0.001	..
27	Narial chini ..	Coco-nut palm sugar (home made).	<i>Cocos nucifera</i>	0.57	0.34	0.10	98.61	0.38	0.036	0.017	..
28	Khajur chini ..	Date-palm sugar (home made)	<i>Phoenix dactylifera</i>	0.46	0.29	0.06	98.84	0.35	0.052	0.027	..
29	Chinia badam ka khali ..	Ground-nut cake	<i>Arachis hypogaea</i>	7.22	40.89	7.43	38.74	2.54	0.213	0.548	3.18
30	Imli ka bia ka gudda ..	Roasted kernel of tamarind seeds.	<i>Tamarindus indicus</i>	9.93	16.13	7.29	64.08	1.62	0.121	0.237	0.95
31	Tar ka chini ..	Palmyra palm sugar (home made).	<i>Borassus flabellifer</i>	0.29	0.37	0.05	98.85	0.44	0.090	0.058	..
32	Sal ka phal	<i>Shorea robusta</i>	9.95	8.35	11.84	66.39	0.97	0.102	0.149	2.50

TABLE—*concl.*

Serial number.	Hindi or local name.	English name.	Latin name.	Moisture, %	Protein, %	Ether extractives, %	Carbohydrates, %	Mineral matter, %	Calcium, %	Phosphorus, %	Crude fibre, %
LEAFY VEGETABLES.—											
33	Agast ka phool	92.91	1.03	0.53	4.36	0.36	0.009	0.005	0.81
34	Bhent ka phool ..	Flowers of water lily	<i>Nymphaea lotus</i>	90.83	1.56	0.58	5.40	0.74	0.029	0.018	0.89
35	Chakwar sag (dried) (lupu) ..	Dried foetid cassia	<i>Cassia tora</i>	9.70	20.68	3.88	43.60	11.75	3.216	0.292	10.39
36	Giria sag	<i>Suaeda nudiflora</i>	89.89	2.10	0.19	2.61	3.88	0.060	0.028	1.03
37	Kanta gendhari sag ..	Thorny pigweed	<i>Amaranthus spinosus</i>	85.08	5.18	0.70	5.04	2.84	10.340	0.084	1.16
38	Sarli sag ..	Tender leaves and stalk	<i>Vauqueria spinosa</i>	70.88	3.97	1.13	15.00	1.55	0.127	0.031	1.47
MILK AND MILK PRODUCTS.—											
39	Chhena (gai ka dudh ka) ..	Casein from cow milk	56.33	14.72	21.97	5.34	1.64	0.497	0.320	..
40	Chhena (bhais ka dudh ka) ..	Casein from buffalo milk	54.10	13.39	23.00	7.94	1.57	0.480	0.277	..

of tamarind seeds are fairly good sources of calcium and are eaten by the poorer-class people in Chota Nagpur when the family stock of grains fall short every year during the rains. *Giria sag* grows on sandy soil in the barren sea coast and its salty taste saves the cost of common salt in its cooking. Consequently, it was found to be widely consumed by the poorer section of the people in Midnapur district during the visit of the senior author (K. M.) there some months ago. The samples of home-made crude sugars were received from one of the home industries associations with a request for analysis. They were analysed as also a sample of commercial (mills made) sugar to study the comparative properties.

SUMMARY.

Two kinds of grain foods, 7 kinds of flesh foods, 5 kinds of fruits, 9 kinds of mushrooms, 9 kinds of miscellaneous foods, 6 kinds of leafy vegetables and 2 kinds of milk products, in all 40 kinds of foods, have been analysed chemically as to their respective protein, fat, carbohydrate, moisture, ash, calcium and phosphorus content. The protein content of the 9 kinds of edible mushrooms varied between 2 to 7 per cent. Meat of mussel and immature shrimps gave high calcium figures. The list of foods included amongst others meat of field rat and bull frog and white ants which were listed as 'food consumed' during dietary survey operations in certain sections of population in Bihar.

The authors are obliged to Rai Bahadur Dr. B. P. Mozoomdar, Director of Public Health, Bihar, for his interest in the work.

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STUDIES ON CALCIUM AND PHOSPHORUS METABOLISM.

Part I.

CALCIUM, PHOSPHORUS AND PHYTIN CONTENTS OF THE DIETS OF KANGRA VALLEY AND THEIR RELATION TO NUTRITION.

BY

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THE occurrence of gross abnormalities of calcium and phosphorus metabolism in the population of Kangra valley, Punjab, is now well recognized. A number of investigations in this area have shown the high incidence of rickets and osteomalacia resulting from this abnormality.

The Public Health Department of the Government of Punjab (Report, Punjab Public Health Department, 1939), after an extensive survey of the diets and nutritional condition of the population of this area, concluded that the diets were ill balanced containing the minimum of protective foods, and were deficient in animal fat, animal protein, calcium and vitamins A, C and D.

Later, the results of a more systematic investigation extending for over two years were published by Wilson and Widdowson (1942). These authors showed that the diets were grossly deficient in calcium, and suggested that this deficiency was aggravated by the presence in the food of large quantities of phytic acid since the diets consisted largely of cereals. The fact that rickets and osteomalacia did not occur in the Central Provinces and Orissa where the diets were similar with respect to calcium and phytic acid content, was explained on the ground that in Kangra valley there existed a superimposed vitamin D deficiency over a low calcium and high phytic acid diet.

The results of both of these admirable and extensive investigations leave a few points which need further investigation. While the exceedingly low calcium level of the diets has been definitely proved, no actual observations on the phytic acid and vitamin D contents of the foodstuffs of the area have been made. It was, therefore, considered desirable to extend the study of this problem further in the light of modern knowledge. It was desirable to determine to what extent the various factors which are known to affect calcium and phosphorus metabolism were of significance in this case. In this paper the calcium and phosphorus ratios of the diets of this area have been studied, and an attempt has been made to investigate the rôle of phytic acid by obtaining information about the *phytic acid* content of samples of food grown in the area. In view of the exceedingly low calcium level of the diets a typical diet of the area has been fed to young rats and its effect upon growth in general and the development of bones has been studied, with the diet as such and after its improvement by the addition of calcium, bringing the calcium and phosphorus ratio within normal limits. At the same time the retention of calcium and phosphorus on a typical diet of the area has been studied in experimental animals.

EXPERIMENTAL.

Certain foodstuffs grown in Kangra valley were obtained and analysed for their calcium, phosphorus and phytin contents. Since the diets of the valley consist largely of cereals and pulses, several samples of these from different areas were examined. Calcium was estimated by the usual method of precipitation as oxalate and titration of the oxalic acid by standard potassium permanganate solution (Peters and van Slyke, 1937); phosphorus

was estimated according to the method of Bodansky, and phytin according to the method described by McCance and Widdowson (1935). The results are shown in Table I. The table also includes available calcium and available phosphorus. These have been calculated by subtracting two-thirds the amount of calcium and phosphorus which will theoretically combine with the amount of phytic acid present, presuming that it is unavailable :—

TABLE I.
Calcium, phosphorus and phytic acid contents of various foods.

Name of the foodstuff.	Where grown.	Total calcium, mg. per cent.	Total phosphorus, mg. per cent.	Total phytin phosphorus, mg. per cent.	Available calcium, mg. per cent.	Available phosphorus, mg. per cent.
Rice (<i>Oryza sativa</i>) ..	Launa (Kangra) ..	12.5	110.0	28.4	6.39	91.07
	Lahore ..	21.5	112.5	23.0	16.54	97.17
	Dharmsala (Kangra)..	20.0	103.0
Wheat (<i>Triticum vulgare</i>).	Launa (Kangra) ..	48.3	315.0
	Lyallpur (228) ..	62.0	312.0
	Lyallpur (D 1) ..	108.0	480.0	68.9	93.19	434.07
	Lahore ..	51.0	311.0	88.9	31.89	251.74
Maize (<i>Zea mays</i>) ..	Launa (Kangra) ..	14.5	214.0
	Dharmsala (Kangra)..	50.0	325.0
	Lahore ..	53.0	352.0	211.4	7.55	211.07
Barley (<i>Hordeum vulgare</i>).	Gorra (Kangra)	186.5
	Lahore ..	52.0	318.0	177.0	13.94	200.00
Bajra (<i>Pennisetum typhoideum</i>).	Lahore ..	208.0	240.0	62.5	194.56	198.34
Black gram (<i>Phaseolus mungo</i>).	Gorra (Kangra) ..	140.0	313.0	166.6	104.31	201.90
	Dharmsala (Kangra)..	135.0	350.0
	Lahore ..	152.0	310.0	138.8	122.16	217.47
Green gram (<i>Phaseolus radiatus</i>).	Gorra (Kangra) ..	105.0	301.0	74.4	89.00	251.40
	Dharmsala (Kangra)..	110.0	325.0
Bengal gram (<i>Cicer arietinum</i>).	Lahore ..	150.0	640.0	49.2	139.42	607.20
	Dharmsala (Kangra)..	141.0	620.0
White gram (<i>Cicer arietinum</i>).	Lahore ..	208.0	240.0
	Gorra (Kangra)	23.5
Kulth (<i>Dolichos biflorus</i>)	Gorra (Kangra) ..	283.2	469.0	125.0	256.32	385.67
	Lahore ..	282.5	450.0	121.0	256.49	369.34
Arhar (<i>Cajanus indicus</i>)	Lahore ..	76.0	436.0	107.5	52.89	364.34
Dal kalan ..	Dharmsala (Kangra)..	67.0	498.0

TABLE I—*concl'd.*

Name of the foodstuff.	Where grown.	Total calcium, mg. per cent.	Total phosphorus, mg. per cent.	Total phytin phosphorus, mg. per cent.	Available calcium, mg. per cent.	Available phosphorus, mg. per cent.
Beans (<i>Vigna catieng</i>)	Launa (Kangra)	31.5
	Dharmsala (Kangra) ..	135.0	509.0
	Lahore ..	138.0	513.0
Red beans ..	Gorra (Kangra)	329.4
	Dharmsala (Kangra) ..	183.0	401.0
	Lahore ..	184.0	444.0	301.0	119.28	243.34
Lobia (<i>Vigna catieng</i>)	Lahore ..	40.0	400.0	107.5	16.89	328.34

It would be seen from Table I that the calcium content of the foods grown in Kangra valley tend to be somewhat lower in most of the cereals and pulses examined. Lander (Report, Punjab Public Health Department, *loc. cit.*) after an analysis of the soils of this valley has stated that calcium content of the soils is 3 to 6 times lower than the figures for Lyallpur and other parts of the Punjab. With respect to the phosphorus content of the foods grown in Kangra and other parts of the Punjab, there does not appear to be much difference. The same is true of the phytin content of the cereals for crops produced in Lahore and Kangra valley.

A number of surveys of the diets of various sections of the population in Kangra valley and neighbouring areas have been reported in the literature, particularly from the studies of the Punjab Public Health Department (*loc. cit.*), and those of Wilson and Widdowson (*loc. cit.*). We have selected 21 of these dietary studies for further investigation of their calcium, phosphorus, phytin, available calcium, and available phosphorus content. The diets selected are shown below :—

Diet.

1. 15 peasant families of village Launa.
2. 15 " " " Gorra.
3. 15 " " " Chilali.
4. 15 " " " Bhadwar.
5. 23 families of Launa village with osteomalacia and rickets.
6. 15 " " " " no osteomalacia and rickets.
7. 10 " " " " osteomalacia and rickets in autumn.
8. 10 " " " " osteomalacia and rickets in spring.
9. 10 " " " " no osteomalacia and rickets in autumn.
10. 10 " " " " no osteomalacia and rickets in spring.
11. 6 individual women with osteomalacia.
12. 6 individual children of mothers with osteomalacia.
13. 10 families of Tharl village with rickets or osteomalacia.
14. 10 families of Tharl village without rickets or osteomalacia.
15. Wheat-eating well-to-do Hindu families of Central Punjab.
16. " middle-class Hindu families of Central Punjab.
17. " poor-class Hindu families of Central Punjab.
18. " middle-class Mohammedan families of Central Punjab.
19. " poor-class Mohammedan families of Central Punjab.
20. " middle-class Sikh families of Central Punjab.
21. " poor-class Sikh families of Central Punjab.

Diets 1 to 4 are from the Report, Punjab Public Health Department (*loc. cit.*) and 5 to 21 are from Wilson and Widdowson (*loc. cit.*). The calcium, phosphorus and phytin

contents of these diets have been re-calculated from our own analyses supplemented where necessary with those of Khullar and Lander (Report, Punjab Public Health Department, *loc. cit.*). From the literature it appears that approximately 60 per cent of the phosphorus present in phytin may be excreted unchanged in the faeces (McCance and Widdowson, *loc. cit.*). Under ordinary conditions, therefore, the phosphorus of the phytin is only available to the extent of about one-third. As Harrison and Mellanby (1939) have pointed out a certain amount of the calcium from the food is also precipitated as calcium phytate, which is again unavailable. Since about one-third of the phosphorus of phytin is available, it can be assumed that calcium phytate is hydrolysed to a limited extent in the body and only about one-third of the calcium from calcium phytate may also be available. On this assumption the available calcium and available phosphorus have been calculated from the total calcium and phosphorus content of these diets. The results are shown in Table II:—

TABLE II.

Total calcium and phosphorus, available calcium and phosphorus, their ratio in the diets of Kangra valley and Central Punjab.

(Per day per consumption unit.)

Dietary number.	Total calcium, mg.	Total phosphorus, mg.	Ca/P ratio.	Total phytin phosphorus, mg.	Available calcium, mg.	Available phosphorus, mg.	Available Ca/P ratio.
1	304	1,515	0.20	358	227	1,276	0.178
2	575	2,784	0.21	447	479	2,486	0.190
3	543	33,160	0.17	951	339	2,526	0.134
4	716	3,150	0.22	782	548	2,629	0.208
5	290	1,990	0.14	623	156	1,575	0.099
6	360	2,160	0.16	728	204	1,675	0.121
7	260	2,050	0.12	703	109	1,581	0.069
8	340	1,890	0.18	614	208	1,480	0.140
9	290	2,260	0.12	1,069	60	1,547	0.039
10	290	2,340	0.12	1,017	71	1,662	0.013
11	240	1,760	0.14	386	157	1,503	0.104
12	220	1,540	0.15	354	144	1,304	0.110
13	290	3,600	0.08	1,664	..	2,491	..
14	300	3,250	0.09	1,493	..	2,255	..
15	1,220	2,250	0.54	421	1,129	1,969	0.570
16	900	1,900	0.47	524	787	1,531	0.507
17	510	1,640	0.37	441	415	1,346	0.308
18	740	1,860	0.40	348	665	1,628	0.408
19	590	1,750	0.30	355	514	1,513	0.340
20	1,050	2,140	0.50	496	943	1,809	0.520
21	770	2,100	0.30	561	649	1,726	0.370

Diets 1 to 14 are of the population groups in Kangra valley, while 15 to 21 represent diets of various sections of the population living in Central Punjab. The calcium contents of Kangra valley diets vary from 220 mg. to 716 mg. per day per consumption unit, the majority of the values approximating to 300 mg. The daily consumption of phosphorus varies from 1,515 mg. to 3,600 mg., while the calcium: phosphorus ratios of the diets are between 0.08 and 0.22. On the other hand, Central Punjab diets show a calcium content varying between 510 mg. and 1,220 mg. and phosphorus content between 1,640 mg. and 2,250 mg. Even in the groups representing low economic levels the calcium consumption is markedly higher than in Kangra valley diets the phosphorus remaining approximately the same.

Consequently, the ratios for these diets are much higher varying between 0.3 and 0.54, for the majority of diets the average being 0.4. At this level of calcium and phosphorus intake there is little incidence of rickets. In Kangra valley diets, however, the calcium contents are low while the phosphorus contents are exceedingly high, leading to a very low calcium : phosphorus ratio. Much of the disturbance, therefore, may arise from the lack of balance between calcium and phosphorus. This point has not been sufficiently stressed by previous workers in this field. The average ratio of calcium : phosphorus of Kangra valley diets is found to be 0.15. This ratio is definitely low for the proper calcification of the bones and for growth. Experimental studies on calcium and phosphorus metabolism have indicated that when calcium or phosphorus is abnormally high neither of them can be retained in the body. It has been found by workers in this field that the range of limits of calcium : phosphorus ratio for normal calcification of the bones appears to lie between 5 and 2 for infants ; between 2 and 1 for older children ; and between 1 and 0.5 for adults. The ratio of the diet in Kangra valley is never higher than 0.2. It is, therefore, to be expected that the calcification of the bones will be markedly hindered with a diet having such a ratio and incidence of rickets and osteomalacia would be very high. Pregnancies which entail still greater strain on the body as regards calcium, precipitate osteomalacia in the majority of women in this area.

In the wheat-eating areas of Central Punjab (diets 15 to 21) which have been included for comparison, the calcium and phosphorus ratio for well-to-do and middle-class families (Hindus) lies between 0.47 and 0.54 which are probably enough for proper calcification. Only in the case of very poor classes both of Mohammedans and of Sikhs, the ratio falls to 0.3 which is definitely low, and rickets and osteomalacia is probably prevented in these areas on account of higher intake of vitamin D occasioned by greater hours of sunshine and outdoor life led by the poorer families of these areas in the Punjab as compared to Kangra valley where the hours of sunshine are much less at certain seasons of the year.

When the phytic acid of the diets is taken into consideration and the ratios of available calcium and available phosphorus for the various diets are calculated, the values tend to be still lower in Kangra valley. Thus, if this represents the true state of their calcium and phosphorus intake the abnormality will be still further aggravated. Considering both the series of ratios, however, the phytic acid contents lower the ratio to the extent of about 10 per cent. The presence of phytic acid, therefore, further aggravates the imbalance of calcium and phosphorus in the diets.

Growth and development of the bones of experimental animals on a typical diet of Kangra valley.

A typical diet of Kangra valley (diet I, described above) was fed to a group of young rats. The animals were divided into two groups one being fed on the typical diet alone and the other on the typical diet supplemented with calcium lactate so as to bring the calcium : phosphorus ratio to 1 : 1. Two such experiments were conducted, one for 11 weeks and the other for 9 weeks. The results are shown in Table III :—

TABLE III.

Group and experimental diet.		Number of animals.	Total time of experiment, weeks.	Average rate of growth, g. per week.	Average food intake per animal per day, g.
<i>Experiment I.</i>					
GROUP 1.	Typical diet of Kangra	6	11	2.40	6.40
GROUP 2.	Typical diet supplemented with calcium lactate.	6	11	3.56	6.50
<i>Experiment II.</i>					
GROUP 1.	Typical diet alone	6	9	1.40	5.00
GROUP 2.	Typical diet supplemented with calcium lactate.	6	9	2.50	5.20

It will be seen from Table III that in both the experiments the average rate of growth of the animals receiving the typical diet of Kangra valley was exceedingly poor. When this diet was supplemented with calcium lactate there was slight improvement in the rate of growth in both the experiments though even as such the growth was much below the normal, being only 25 to 35 per cent of what is generally obtained on a good diet. This diet is evidently lacking in other essential factors also, which act as limiting factors for growth. It is clear, however, that by itself the diet is very poor.

The effect of these diets was also studied on the development of the bones. At the conclusion of the experiments summarized in Table III all the animals were killed and the entire skeleton of each rat was removed by the usual method of proteinase digestion. The skeletons were thoroughly washed, dried, defatted with ether and ashed in a silica crucible in the muffle furnace. Total calcium and phosphorus contents of the ash in the case of each skeleton were determined by the usual methods. The results of the weights of the bones, bone ash, the degree of calcification showing the average of each group are shown in Table IV:—

TABLE IV.

Calcium and phosphorus content of the bones of rats kept on Kangra valley diet alone and after supplementing it with calcium lactate.

Group and experimental diet.		Weight of bones, g.	Weight of ash, g.	Percentage of ash.	A/R.*	Total calcium, mg.	Percentage of calcium.	Total phosphorus, mg.	Percentage of phosphorus.
<i>Experiment I.</i>									
GROUP 1.	Typical diet of Kangra ..	1.364	0.670	49.1	0.946	67.66	4.91	103.1	7.522
GROUP 2.	Typical diet supplemented with calcium lactate.	2.033	1.110	57.5	1.140	141.96	6.852	188.32	9.330
<i>Experiment II.</i>									
GROUP 1.	Typical diet alone ..	0.878	0.430	48.88	0.960	60.06	6.876	79.96	9.090
GROUP 2.	Typical diet supplemented with calcium lactate.	1.478	0.785	53.30	1.130	105.16	7.250	139.24	9.58

* See note below.

Table IV shows that the values for average weight of bones, amount of bone ash, percentage of bone ash are all lower for the animals fed on the typical diet of Kangra valley than those whose diet was supplemented with extra calcium. The degree of calcification represented by $A/R = \frac{\text{weight of ash}}{\text{weight of bones} - \text{weight of ash}}$ was also lower for the first group of animals in both the experiments. There was only 5.8 per cent increase in the percentage of bone ash on feeding extra calcium.

Calcium and phosphorus balance and retention on a typical diet of Kangra valley.

A further metabolism experiment was undertaken to see the retention of calcium on a typical diet of Kangra valley (diet 1, described above). Each experiment was carried out on six adult rats of about six months of age and weighing between 100 g. and 120 g. After the preliminary period of three days on the experimental diet the urine and faeces were collected during a subsequent period of three days, the food intake being also determined.

The weights of the rats during the experiment showed little change. Three such experiments were performed and the results are shown in Table V :—

TABLE V.

Average metabolism data showing the balance and retention of calcium and phosphorus on a typical diet of Kangra valley and the same diet supplemented with calcium.

Group and experimental diet.	Period.	INTAKE.		OUTPUT OF CA.		OUTPUT OF P.		BALANCE.	
		Ca, mg.	P, mg.	Urine, mg.	Fæces, mg.	Urine, mg.	Fæces, mg.	Ca.	P.
<i>Experiment I.</i>									
GROUP 1. Kangra valley diet.	3 days	10.17	50.40	3.17	10.95	23.13	18.23	— 3.96	+ 9.03
GROUP 2. Supplemented diet.	„	49.00	49.00	1.50	23.73	17.90	20.76	+23.83	+10.33
<i>Experiment II.</i>									
GROUP 1. Kangra valley diet.	„	17.17	85.74	2.80	20.40	22.05	25.80	— 6.02	+37.86
GROUP 2. Supplemented diet.	„	81.20	81.20	2.83	21.56	23.20	28.58	+53.46	+29.41
<i>Experiment III.</i>									
GROUP 1. Kangra valley diet.	„	10.63	53.20	5.02	7.77	26.79	16.36	—21.15	+10.43
GROUP 2. Supplemented diet.	„	52.80	52.80	4.02	22.60	24.06	18.46	+26.18	+10.26

The results of these experiments clearly indicate that there was a negative balance of calcium when the typical diet of Kangra valley alone was fed. When the diet was supplemented with calcium there was retention of calcium. For phosphorus, however, the balance was positive in all the groups. This experiment lends further support to the contention that the diet was ill balanced with regard to calcium and phosphorus resulting in the excretion of calcium, ultimately precipitating osteomalacia.

SUMMARY.

1. The investigations described in this paper relate to diets in Kangra valley, Punjab, where abnormalities of calcium and phosphorus metabolism occur to a very marked degree resulting in a high incidence of rickets and osteomalacia. Some typical foods of Kangra valley were analysed for calcium, phosphorus and phytin contents and on the basis of those analyses the average diets of 14 different groups of the population of the valley were studied with regard to their calcium and phosphorus contents. The calcium content was low and phosphorus exceedingly high showing ratios of calcium : phosphorus between 0.08 and 0.22 for the various diets. This ratio is considered to be definitely low for proper calcification. For comparison seven diets of population group from Central Punjab are also shown.

2. The rôle of phytic acid in the nutrition of the population of this area has also been investigated. From the phytic acid content of the foods grown in the area the available calcium and phosphorus of the diet, and the ratio of available calcium : available phosphorus have been calculated. This ratio is about 10 per cent lower than the ratio based on total

calcium and total phosphorus. The presence of phytic acid therefore significantly aggravates the imbalance of minerals in the diets of these population groups.

3. A typical diet of Kangra valley was fed to young animals to study its effect upon (a) general growth and (b) development of bones. The growth on this diet was exceedingly poor, there being slight improvement when the diet was supplemented with calcium. The rate of growth even on the improved diet was only 25 to 35 per cent of the normal showing that there were other limiting factors involved. The skeletons of these rats were examined for their ash, calcium and phosphorus contents. The degree of calcification was poor in the case of animals fed on typical diet of Kangra valley, while the percentage of calcium and phosphorus in the bone ash together with the degree of calcification considerably improved for the animals whose diet was supplemented with extra calcium.

4. In another group of 18 rats on a typical diet of Kangra valley calcium balance was studied. All the animals were found to be on a negative calcium balance. On adding calcium to the diet so as to bring the ratio of calcium : phosphorus as 1 : 1, retention of calcium began to take place.

From these studies it is concluded that one of the chief abnormalities in the diets of the population of this area is the lack of balance of the minerals calcium and phosphorus.

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STUDIES ON CALCIUM AND PHOSPHORUS METABOLISM.

Part II.

BLOOD-SERUM CALCIUM, PHOSPHORUS AND PHOSPHATASE OF THE POPULATION OF KANGRA VALLEY.

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THE high incidence of rickets and osteomalacia in Kangra valley, Punjab, and the gross deficiency of food with respect to calcium has been fully established by the investigations of the Punjab Public Health Department (1939) and of Wilson and Widdowson (1942). In an earlier investigation (Ahmad, Dutt and Narang, 1945), it was shown that the ratio of total calcium : phosphorus in the common diets of this area was very low and was of the order of 0.2. If the phytin content of the diets was taken into consideration this ratio was still further lowered. The deficiency of the diet was further confirmed by feeding experiments on the animals, and even when the diet was corrected with respect to calcium and phosphorus level, and the balance of these minerals, the growth of animals was by no means normal, indicating the coexistence of other abnormalities.

The object of the present investigation was to study the blood conditions of the people in respect of calcium, phosphorus and phosphatase contents and to relate them to earlier findings. Blood examinations of the calcium, phosphorus and phosphatase contents were made on the patients attending the Civil Dispensary and Zenana Mission Hospital, Palampur. It was from these patients that normals as well as cases of rickets and osteomalacia were selected. The cases grouped as normals were those who had no ostensible signs of any pathological condition, and attended the hospital for only petty ailments. The selection of the rickets and osteomalacia cases was made on a clinical basis. In the table, where information regarding blood-serum calcium, phosphorus and phosphatase for this class of patients is set out, the severity of each case is also indicated. This degree of severity was assessed exclusively on the clinical symptoms and is based on the opinion of the medical officer in-charge of the dispensary.

METHOD.

Blood-serum calcium was determined by the usual method of precipitating it as calcium oxalate, and titrating the combined oxalic acid with standard potassium permanganate solution as modified by Clark and Collip (1925).

Phosphorus and phosphatase were determined by the modified method of Bodansky (1937).

In Tables I to V the values of calcium, phosphorus and phosphatase for 100 samples of blood are shown for the different groups, viz. normals, ricketic and osteomalacic.

TABLE I.

*Calcium, phosphorus and phosphatase contents of blood.
Normal adult males (10 cases).*

Number.	Age (years).	Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1	15	8.51	4.50	2.3
2	35	8.20	4.80	3.1
3	27	8.85	4.56	1.9
4	19	9.12	4.38	2.5
5	48	9.03	4.21	1.5
6	36	8.88	5.23	4.0
7	29	9.35	4.75	1.78
8	56	9.26	5.00	1.6
9	64	9.12	4.92	1.8
10	41	9.40	4.78	2.4

TABLE II.

Normal adult females (20 cases).

Number.	Age (years).	Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1	23	8.3	4.0	3.1
2	17	8.2	4.25	2.9
3	29	8.55	4.6	2.2
4	35	9.24	4.8	1.8
5	30	9.0	4.7	1.6
6	22	8.45	4.3	3.7
7	33	8.12	4.4	1.9
8	18	8.35	4.5	2.1
9	45	8.21	5.0	2.0
10	35	8.45	4.2	3.1
11	24	8.17	4.4	2.6
12	39	8.85	4.5	1.8
13	35	8.24	4.7	3.2
14	18	8.35	3.9	2.7
15	25	8.09	4.6	2.1
16	54	8.22	4.9	2.4
17	64	8.75	4.5	1.9
18	27	8.16	4.2	3.7
19	25	8.53	4.4	2.0
20	31	8.24	4.6	2.2

TABLE III-A.
Normal female children (6 cases).

Number.	Age (years).	Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1	5	8.80	4.2	9.3
2	4	9.12	4.8	10.2
3	8	8.78	4.65	8.7
4	6	8.75	4.85	11.3
5	8	8.15	4.32	13.0
6	7	8.55	5.0	6.32

TABLE III-B.
Normal healthy male children (17 cases).

Number.	Age (years).	Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1	7	8.60	3.9	6.6
2	11	8.75	4.37	5.4
3	9	9.22	4.32	7.85
4	5	8.35	4.74	9.26
5	10	8.42	5.0	8.8
6	8	8.31	4.38	9.7
7	12	8.85	4.85	6.32
8	6	9.08	4.74	10.40
9	9	8.24	4.55	7.75
10	13	8.72	4.72	5.95
11	8	9.00	5.20	8.46
12	5	8.61	4.36	7.32
13	10	8.15	4.00	11.85
14	4	8.52	4.71	9.35
15	6	9.25	4.85	8.67
16	6	8.24	4.10	10.38
17	12	8.70	3.81	6.85

TABLE IV-A.
Rachitic male children (13 cases before treatment).

Number.	Age (years).	Degree of rickets.	Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1	5	++	7.10	4.04	11.3
2	4	+++	6.88	3.55	15.6
3	4	+	7.08	4.10	10.5
4	9	+++	6.81	3.75	13.7
5	6	+	7.20	4.	10.2
6	8	+	7.16	4.52	10.7
7	5	+	6.95	4.05	11.3
8	5	+++	6.68	3.52	16.3
9	7	++	7.00	4.03	13.5
10	7	++	6.85	4.12	14.1
11	6	+++	6.21	4.60	24.5
12	8	+++	6.51	4.28	15.8
13	4	++	6.89	4.18	12.8

TABLE IV-B.
Rachitic female children (12 cases before treatment).

Number.	Age (years).	Degree of rickets.	Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1	3	++	7.12	3.72	14.8
2	2	++	6.74	3.45	17.35
3	7	++	6.90	3.95	9.8
4	7	++	6.75	3.92	14.8
5	9	++	6.92	3.96	12.9
6	7	+	7.20	4.53	12.5
7	7	+	7.25	4.37	10.7
8	12	+++	6.35	4.15	17.7
9	6	+	7.05	4.83	12.7
10	6	+	7.10	4.80	13.2
11	7	++	6.91	4.75	14.8
12	5	+++	6.68	3.75	20.3

TABLE V.

Women with osteomalacia (25 cases before treatment).

Number.	Age (years).	Degree of rickets.	Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1	41	++	6.85	5.02	7.2
2	25	+++	6.50	4.80	9.5
3	35	+	7.03	4.75	5.25
4	22	++	6.90	4.66	6.5
5	26	+	6.95	4.35	4.8
6	19	+	7.20	4.00	5.5
7	32	++	6.92	4.50	7.3
8	27	++	6.81	4.55	6.9
9	24	+	7.00	4.34	5.20
10	36	++	6.78	4.20	8.4
11	28	+++	6.42	4.05	10.7
12	25	+	7.10	4.72	4.85
13	24	++	6.88	4.53	5.9
14	26	+	6.95	4.38	5.0
15	35	+	7.15	4.58	6.3
16	29	++	6.78	4.16	6.5
17	22	+++	6.18	4.32	9.2
18	35	++	6.91	4.54	7.1
19	20	++	6.81	4.00	6.4
20	24	+	7.00	4.60	5.2
21	19	++	6.85	4.35	6.8
22	34	++	6.80	4.22	5.7
23	30	++	6.71	4.56	8.4
24	35	++	6.79	4.00	7.1
25	25	+++	6.45	3.90	8.4

STATISTICAL ANALYSIS OF THE DATA.

The mean values of calcium, phosphorus and phosphatase as deduced from Tables I and II for the 10 adult healthy males and 20 healthy adult females respectively, together with the variances of each series, are shown in Table VI:—

TABLE VI.

		Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1. Mean values.				
(a)	Normal adult males ..	8.97	4.72	2.29
(b)	Normal adult females ..	8.42	4.48	2.45
2. Variance.				
(a)	Normal adult males ..	0.14	0.09	0.60
(b)	Normal adult females ..	0.10	0.08	0.41

The significance of the difference existing between the mean values of the two sexes has been tested by the usual Fisher's (1938) 't' test. Sex difference is found to be statistically significant only in respect of calcium and phosphorus contents of the serum. But, whilst in respect of calcium, the difference in the mean values of the two sexes is highly significant, the phosphorus content is not so markedly different; being significant at the 5 per cent but not at the 1 per cent probability levels.

In so far as the degree of variability in the male and female series is concerned in no case is any sex difference established statistically.

Mean values for normal children similar to those set out in Table VI for adults are presented in Table VII. The number of normal male children was 17 and that of the normal female children 6. It may be mentioned that all cases below 13 years of age were classed as children. The ages of the adults ranged from 15 to 65 years in the different tables set out in the text.

TABLE VII.

	Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1. <i>Mean values.</i>			
(a) Normal male children ..	8.65	4.51	8.31
(b) Normal female children ..	8.62	4.56	9.56
2. - <i>Variance.</i>			
(a) Normal male children ..	0.19	0.16	3.15
(b) Normal female children ..	0.09	0.13	0.48

None of the three variances for male children shown in Table VII differ from the corresponding variances for females.

In respect of mean values, comparisons were instituted not only in respect of sex but also between children and adults of the same sex. It was found that amongst children, no sex differences could be established in regard to the mean values of any of the three constituents of the blood. When comparison is made between adults and children of the same sex, a highly significant difference is established only in regard to the mean phosphatase values; the mean phosphatase value for male adults being 2.29 mg. as compared with that of 8.31 mg. for male children. Similarly, for the female adults, the mean phosphatase is 2.45 mg. as against a value of 9.56 mg. for the female children. Age does not, however, seem to produce significant variations when we consider the mean phosphorus content of blood serum either for males or females. In respect of calcium, only the male children show a significant difference from the male adults. This difference is significant at 5 per cent level but not at the 1 per cent probability level. Females fail to show this difference to a significant extent. Thus, whilst among normal females age does not seem to make any appreciable difference in the serum calcium, there is an indication that an increase occurs in the mean calcium level of adult males as compared with that of the male children. The mean calcium content among male children is 8.65 mg. as compared with a mean of 8.97 mg. for male adults. The corresponding values for females are 8.62 mg. for children and 8.42 mg. for the adults.

In Table VIII the mean values and variances for rachitic children (male and female separately) and for osteomalacia cases are set out. The numbers of cases falling in the three groups, viz. rachitic male children, rachitic female children and osteomalacia cases, were 13, 12 and 25, respectively.

TABLE VIII.

	Calcium per 100 c.c. serum, mg.	Phosphorus per 100 c.c. serum, mg.	Phosphatase per 100 c.c. serum, B. units.
1. <i>Mean values.</i>			
(a) Rachitic male children ..	6.87	4.08	13.87
(b) Rachitic female children ..	6.91	4.20	14.29
(c) Osteomalacia cases ..	6.83	4.41	6.81
2. <i>Variance.</i>			
(a) Rachitic male children ..	0.08	0.10	14.59
(b) Rachitic female children ..	0.08	0.10	14.59
(c) Osteomalacia cases ..	0.06	0.08	2.46

The amount of variability associated with the calcium and phosphorus contents is almost similar for the male and female children and for the osteomalacia cases. The individual values of phosphatase, however, show markedly different degrees of variability; thus, whilst the variance for the osteomalacia cases is 2.46, that of rachitic female children is 3.4 times this value, and that of the rachitic male children is 5.9 times. Both these variances are significantly higher than that for osteomalacia cases:

The results of the different comparisons made between the mean values for rachitic children and osteomalacia cases and of the corresponding normal cases are summarized below:—

Between rachitic male and female children no difference seems to exist in respect of any of the three constituents. In the earlier ages, therefore, among rachitic children sex makes no difference. But, compared with the normals, in so far as the male rachitic children are concerned, the mean values of all the three constituents, viz. calcium, phosphorus and phosphatase, are significantly different from those for the normal children. Female rachitic children, however, whilst differing significantly from the normal female children in respect of phosphatase and calcium values, do not show any material difference in respect of phosphorus content of blood serum. At the higher ages also, when comparison is made between adult normal females and osteomalacia cases, there is no evidence that the mean values of phosphorus for the two groups are different. At these ages the other two constituents, viz. calcium and phosphatase, are, however, both significantly different, in the osteomalacia group as compared with the normal women. The calcium values for osteomalacia cases are on the average significantly lower and the phosphatase mean values higher than those of the normal female adults.

Age has been shown to have its effect in regard to phosphatase values. For this reason it is necessary to test whether the age distribution of adult normal females is different from that of osteomalacia cases. The number of cases in the two groups are 20 and 25 and, in broad age-groups, the following frequency distributions are obtained:—

TABLE IX.

Age distribution.	Normal adult females.	Osteomalacia cases.
Below 25 ..	6	8
25 to 35 ..	7	11
35 and over ..	7	6
TOTAL ..	20	25

The χ^2 for this table is as low as 0.7 compared with the values of 5.99 at the 5 per cent level of significance. The two age distributions cannot, therefore, be regarded as dissimilar. Further, the mean age of the normal female adult group is 31.45 years as compared with 27.92 years for osteomalacia cases. The value of 't' obtained from the difference of these two mean ages is 1.28 which again does not show any significance. It is, therefore, reasonable to conclude that whatever significant results have been obtained in the foregoing analysis have not arisen from any difference in the age constitution of the two groups of normal female adults and women with osteomalacia.

DISCUSSION.

Perusal of Tables I, II, III-A and III-B shows that although blood-serum phosphorus and phosphatase vary within normal limits there was positive deficiency in respect of calcium. Normal male adults are just on the border line, whereas all women with the exception of 2 in the series of 20 (Table II) showed low values for blood-serum calcium. This is due according to Ahmad *et al.* (*loc. cit.*) to (a) a very poor intake of calcium in their diet, and (b) a very unfavourable ratio of calcium and phosphorus in the diet which depletes the body further of its calcium reserves.

Tables IV-A, IV-B and V give the results of analyses of the pathological cases examined. It is evident that in these cases the condition of rickets and osteomalacia has been precipitated by a further fall in the blood calcium, whereas the blood-serum phosphorus and phosphatase have varied as in the healthy persons within the normal limits. These findings suggest that the patients suffered from high phosphorus and low calcium rickets. The rachitogenic action of their diet which consists chiefly of cereals may have been further enhanced by the deficiency of fats in their diets. McDougal (1938) and Palmer and Mottram (1939) have shown that the calcium from the cereals diet is absorbed to a greater extent because of the calcium soaps formed with the fatty acids, while in the absence of fats calcium combines with phytin or its decomposition products to form an insoluble compound which cannot be absorbed. This may explain why the comparatively richer class of people are free from these symptoms who have a higher dietary intake of fat.

Phosphatase has been found to be considerably elevated in rickets (Robison, 1923; Kay, 1930, 1932). Stevenson, Morris, Pedan and Small (1937) have suggested that the level of plasma phosphatase could be usefully employed as an index of the degree of rickets. They further suggested that in the diagnosis of rickets the estimation of phosphatase provided a sensitive test and showed the very early presence of the disease where even the x-ray failed. But our observations (Tables IV-A and IV-B) show that although other clinical symptoms of rickets were present in the patients examined, the serum-phosphatase values ranged within normal limits, in 5 cases out of 13 among the male children and 2 cases out of 12 among the female children. Similarly, in the osteomalacic women the values although higher than the normal healthy women are not of the same degree of magnitude as recorded in the literature. The only explanation from the data presented can be that work reported in literature has more or less been limited to high calcium, low phosphorus rickets whether on human beings or the experimental animals. Phosphatase, being an enzyme which is primarily concerned with glycolysis, will only be increased where there is a deficiency of phosphorus in the body. In phosphorus deficiency rickets it becomes more active and may remove phosphorus from the skeleton, for the more important functions of the body.

The common deformities met with in the patients studied were: Ankylosed knee joints, legs bent, pigeon chest and hunch back. For illustration only two photographs of one patient are included which show the condition of a woman aged 35, who was originally quite normal, had 4 normal pregnancies, then became osteomalacic, began to develop a hunch back and pigeon chest (Plate IV). Her legs and arms became bent like bows and arches. She is now a total wreck, all huddled up and unable even to crawl. She experienced a lot of torture in placing her legs and arms in the position shown. Several other similar cases were seen.

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PLATE IV.



A typical case of an osteomalacic woman.

To Dr. M. Moosa Khan, Nutrition Officer, Department of Public Health, Punjab, for selecting some of the cases reported in the paper, thanks are herewith tendered. Thanks are also due to Dr. B. L. Malhotra, District Leprosy Officer, Kangra, for useful suggestions given during the study.

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STUDIES ON CALCIUM AND PHOSPHORUS METABOLISM.

Part III.

HEPATIC INEFFICIENCY AND RICKETS.

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A NUMBER of investigators have reported cases of rickets which did not respond to moderately high doses of vitamin D, and massive doses of vitamin D had to be administered before effective results could be obtained (Bawkin, Bodansky and Schorr, 1940; Albright, Butler and Bloomberg, 1937; Park and Elliot, 1938). Gill (1939) described 4 cases of rickets in none of which he could obtain any evidence of malnutrition, lack of sunlight, calcium drain due to hyperparathyroidism, coeliac or renal disease, and in none of which did any of the known forms of therapy given over a period of several years produce evidence of healing:

Greaves and Schmidt (1933) and Gerstenberger (1935) observe that in the absence of bile vitamin D was not absorbed from the gastro-intestinal tract. Buchbinder and Kern (1927, 1928, 1928a) in their experimental work on the obstruction of the common bile duct in animals found that they developed after 70 to 80 days a high degree of rarefaction and other bony changes, showing that bile could indirectly affect the bones. Heymann (1937, 1938, 1938a) while studying absorption, excretion and the mode of action of vitamin D observed that the anti-rachitic efficiency of intramuscularly injected vitamin D decreased considerably in ricketic rats in which biliary cirrhosis, or degenerative changes of the liver had been produced. This author confirmed the observation of Gerstenberger (*loc. cit.*) that impaired liver function was responsible for the decreased anti-rachitic efficiency of vitamin D.

The enzyme phosphatase has been found to be intimately connected with bone-formation (Robison, 1923; Kay, 1930). Kay (*loc. cit.*) observed that blood-serum phosphatase was considerably elevated in a group of pathologic conditions affecting the bony tissues, such as local bone atrophy, generalized osteoporosis, osteomalacia, rickets and Paget's disease. Morris, Stevenson, Pedan and Small (1937) stressed the significance of plasma phosphatase in the diagnosis and prognosis of rickets. It was stated that plasma-phosphatase determination could detect the earliest manifestation of rickets, where other methods of differential diagnosis and even x-ray failed. The high level of blood-serum phosphatase in rickets has since been observed by a number of investigators. On the other hand it has also been found that the blood-plasma phosphatase was elevated in cases of liver injury and very striking results were obtained in obstructive jaundice (Bodansky and Jaffe, 1934; Armstrong, King and Harris, 1935; Roberts, 1930; Greene, Shuttack and Kaplowitz, 1934; Freeman, Pingchen and Ivy, 1938; Sehra, Chopra and Mukerji, 1941).

All this work points to a relationship between liver function, blood-serum phosphatase and bone development. Of particular interest is the rise in blood-serum phosphatase in pathological conditions of the bony tissues, and in the cases of hepatic inefficiency. It was considered desirable to study further (a) the possible influence of liver inefficiency created artificially by the administration of hepatotoxic drugs of the type of carbon tetrachloride on the calcification of the bones and (b) the origin of the enzyme and its possible functions.

EXPERIMENTAL.

Experiments were carried out on guinea-pigs. Young healthy animals were kept in metabolism cages on a more or less constant colony diet consisting of gram, bran and leafy

vegetables, and their weights were recorded twice weekly. No attempt was, however, made to keep them under controlled conditions of light. The animals were divided into three groups:

- (i) A group of 5 animals as normal controls.
- (ii) A group of 5 animals which received a rachitogenic diet.
- (iii) A group of 5 animals which were fed carbon tetrachloride.

The batch of 5 animals which received the rachitogenic diet was kept in a darkened room. The rachitogenic diet consisted of:—

Yellow maize	60	parts.
Whole wheat	25	parts.
Gelatine	10	parts.
Calcium carbonate	3	parts.
Common salt	1	part.

The third group were fed carbon tetrachloride, 0.2 c.c. per kilo body-weight, by means of a stomach tube twice weekly. The weights of the animals were regularly recorded. Whenever the animals showed toxic symptoms or a tendency of sudden drop in weight the administration of the drug was discontinued for a few days, and started again when the animals regained their weights. The drug was regularly administered for two months, after which the animals were killed and their bones examined for rickets by line-test and by bone-ash methods. The results are summarized in Tables I, II and III:—

TABLE I.
Group I.—Normal controls.

Number.	Weight of bone.	Weight of bone ash.	Percentage of bone ash.	A/R* (degree of calcification).
1	4.9216	3.2613	66.2	1.96
2	5.7860	3.6200	62.5	1.72
3	5.1970	3.5200	67.7	2.90
4	5.5060	3.5510	64.4	1.81
5	5.2458	3.5731	68.1	2.13

TABLE II.
Group II.—Rachitic control animals.

Number.	Weight of bone.	Weight of bone ash.	Percentage of bone ash.	A/R* (degree of calcification).
1	1.4620	0.9000	61.50	1.60
2	2.1365	1.0965	51.30	1.05
3	1.6711	0.9021	53.80	1.17
4	2.1830	1.2332	56.02	1.30
5	1.2706	0.6818	53.54	1.15

*Degree of calcification A/R = $\frac{\text{Weight of the bone ash}}{\text{Weight of the defatted bones} - \text{weight of the bone ash}}$

TABLE III.

Group III.—Experimental animals fed CCl₄ for one month.

Number.	Number of feeds of CCl ₄ .	Weight of bones.	Weight of ash.	Percentage of ash.	A/R.
1	5	1.2500	0.7306	58.40	1.40
2	4	1.9812	1.0807	54.50	1.20
3	4	1.4313	0.9250	64.60	1.83
4	6	1.0600	0.6395	63.80	1.52
5	8	0.9850	0.6148	62.40	1.66

The results summarized in the above tables show that in the animals of group II, namely in the rachitic controls, both the percentage bone ash and degree of calcification were definitely lower than those of group I, the normal controls. In animals of group III the percentage of bone ash was not so low when compared with animals of group I, but the degree of calcification was significantly lower than that obtained with the normal animals. Excepting the third animal in Table III, all the other animals in this group which were fed carbon tetrachloride, show a poorer degree of calcification of their bones. The values of A/R in these animals are comparable with those of rachitic controls shown in Table II. That this impaired calcification of the bones in the animals fed carbon tetrachloride was actually due to the formation of rickets or merely decalcification of the bones could not be definitely ascertained. All the 5 animals gave negative line test for rickets. For want of x-ray facilities it could not be definitely decided whether this impaired calcification was rachitic or of the type of osteoporosis or merely decalcification of the bones.

This experiment was again repeated on another group of 12 animals. All the animals received the normal stock diet of the laboratory, which consisted of gram, bran and leafy vegetables for the first month. The animals were fed carbon tetrachloride as usual for a total period of two months. Four animals, however, at the end of one month received a modified diet in which gram was replaced by oats. The results of this experiment are shown in Tables IV and V:—

TABLE IV.

Number.	Number of feeds of CCl ₄ .	Weight of bones.	Weight of ash.	Percentage of ash.	A/R.
1	12	0.9266	0.5835	62.90	1.70
2	13	0.9921	0.6350	64.14	1.78
3	12	0.8213	0.5289	64.50	1.82
4	14	0.7740	0.4922	63.50	1.74
5	12	1.0812	0.6904	63.88	1.77
6	13	1.0121	0.6465	63.88	1.77
7	12	0.9750	0.6052	62.05	1.64
8	11	1.9658	1.2510	63.60	1.75

TABLE V.

Number.	Number of feeds of CCl ₄ .	Weight of bones.	Weight of bone ash:	Percentage of bone ash.	A/R.
1	12	2.4876	1.4075	56.60	1.30
2	12	1.1402	0.6692	58.77	1.43
3	13	0.7910	0.4800	60.70	1.55
4	11	1.2308	0.7691	62.60	1.61

The results summarized above again show a significantly lower bone ash as well as A/R values in contrast to normal controls presented in Table I. The values of the bone ash shown in Table V, i.e. of the group of animals fed on the oats diet in addition to the hepatotoxic drug showed a much greater marked lowering of the percentage of bone ash, as well as lowering in the degree of calcification. Excepting animal No. 1 in Table V, which showed only slight positive, all the animals gave negative line test for rickets. It is, however, obvious that something has gone wrong with the process of calcification.

In order to test whether the results recorded in Tables III, IV and V were statistically different from those in Table I, their variances and values of 't' have been determined. The values of A/R on comparison between Tables I and III, show that for 9 degrees of freedom the value of 't' comes out to be 3.15 indicating that the two sets of animals show a statistical difference at 2% level. Comparison of Tables I and IV shows that the value of 't' is 3.3894 which is significant at 1% level for the degree of calcification.

DISCUSSION.

It has been shown by Freeman, Pingchen and Ivy (*loc. cit.*) and Sehra, Chopra and Mukerji (*loc. cit.*) that in practically all types of liver injury artificially induced, whether by hepatotoxic drugs, leptospiral infection, partial or complete obstruction of the common bile duct, an increase in blood-serum phosphatase level occurs. The observations of Kay (*loc. cit.*) and Robison (*loc. cit.*) again show that the serum-phosphatase values are increased in a group of pathologic conditions affecting the formation of the bones. As a result of the administration of carbon tetrachloride, in our experiments, to the animals there occurred a rise in the level of blood-serum phosphatase and it is, therefore, possible that the rise in phosphatase has produced the change in the calcification of the bones. It is probable that the level of enzyme phosphatase in the tissues, which is concerned with the breakdown of the complex organic phosphates into simple inorganic salts, is intimately connected with the function of the liver. An increase in the phosphatase consequent to the liver damage leads to a greater production of inorganic salts in the blood and their increased excretion. This process may lead to a deficiency of certain phosphorus compounds essential for the precipitation of the calcium salts at the site of bone-formation. This process may retard calcification as such or even help in decalcification mechanism by upsetting the local condition. At the same time an increase in phosphatase at the site of bone-formation will lead to disturbance in the equilibrium between different mineral salts, which are responsible for the effective deposition of calcium phosphate and calcium carbonate. Cases are on record that when the serum-phosphatase values have remained high for a pretty long time as in chronic obstructive jaundice, skeletal deformities have occurred as the result of that (Francis Braid, 1939). It has been reported that in such cases vitamin D in ordinary therapeutic doses was not effective. Large amounts of vitamin D had to be administered to effectively lower the phosphatase level and produce conditions favourable to calcification. Further support to this view has been provided by the experiments of Correl and Wise (1939) who have shown that vitamin D has an inhibitory effect on the action of phosphatase and the efficiency of the anti-rachitic vitamin is proportional to the fall in phosphatase level. Unless sufficiently high doses of vitamin D are administered which will bring

down the level of phosphatase to the normal limits the anti-rachitic effect of vitamin D is not exhibited.

It appears, therefore, that the enzyme phosphatase and the functions of the liver are intimately related. Guttman *et al.* (1940), observing an increase in phosphatase in almost all cases of skeletal diseases where no involvement of the liver was present, seem to believe that liver has no relation to phosphatase. As a matter of fact in both conditions phosphatase levels are affected. Increase in phosphatase seem to occur as a result of changes in the process of glycolysis. Liver damage markedly affects this process. In skeletal diseases the process of glycolysis is affected indirectly by upsetting the mineral balance. In infections there is a local rise in the level of serum phosphatase which rises as the result of the effect of those infections upon the process of glycolysis. Due to local increase in phosphatase, the enzyme at times leaks into the blood stream and raises the value of phosphatase in the blood serum. It may not be out of place to mention here that there are cases described by Woodard and Higginbotham (1941) of different types of osteogenic sarcomas where the blood-serum phosphatase values were only normal, and some osteogenic sarcomas produce phosphatase which enters the circulation and can be measured in the serum.

Further work is in progress to study as to what type of changes take place in the calcification of the bones, by the damage to the liver.

SUMMARY.

1. Bone-ash determination and degree of calcification have been studied in guinea-pigs which were fed carbon tetrachloride to produce liver damage. It has been observed that the degree of calcification is lower than normal controls, and that this difference is statistically significant.
2. The addition of oats to the normal stock diet had an additive effect upon the decalcifying action which is the result of the liver damage.
3. The importance of phosphatase and its possible rôle in bringing about skeletal changes is discussed.

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DENTAL AND OSSEOUS CHANGES IN SPONTANEOUS FLUOROSIS IN RATS.

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CHANGES in bones and teeth of rats due to experimental fluoride poisoning have been studied by numerous investigators (McCollum *et al.*, 1925 ; Schour and Smith, 1934 ; Phillips and Lamb, 1934 ; Sutro, 1935 ; Roholm, 1937). The present paper compares such lesions in experimental animals with pathological changes in adult rats suffering from 'spontaneous' fluoride poisoning. The available literature contains no reference to this disease occurring naturally in these animals.

Clinical history and symptoms of affected rats.

The affected rats showing protruded incisor teeth were received from the Director, Imperial Agricultural Research Institute, New Delhi. In a breeding stock of 80, six rats (5 females and one male), aged 6 to 10 months, were found to be affected.

The stock diet was composed of : sprouted gram 680 g., wheat bran 200 g., barley malt 480 g., milk 5 lb., vegetables 1½ lb. and water *ad lib.* for a stock of 74 rats. Green vegetables given were different every day.

The first series of cases which occurred in June 1942 were reported to have shown improvement when put on a diet from which barley malt was eliminated. This modified diet, however, did not seem to prevent the recurrence of the disease in the same rats during the summer of 1943.

All the affected rats showed ruffled and coarse hair and assumed a crouching position. The gait was unaltered but the animals were less active than normal. The incisor teeth in the first series of cases showed only slight protrusion (Plate V, fig. 1) with an irregular colour of the enamel and with the cutting edges blunter and more spatulate than normal, while in the second series of cases the normal orange-brown translucent enamel of the incisors was replaced by streaks and spots of chalky-white enamel (Plate V, fig. 2). At places this was transformed into dull-white diffuse areas, with a coarse and corroded surface. In one rat the overgrowth of the upper incisors had resulted in an extreme backward curvature tending to form complete circles with the points of the teeth thrust against the zygomatic arches on either side. The lower incisors were also elongated and showed considerable lateral deviation.

Biochemical.

The fluorine contents of food and water, as well as of bone samples from three cases of the first series, are given below :—

Food and water.

1. Gram	4.32	mg.	per	kg.	} On dry matter basis.
2. Barley	3.38	"	"	"	
3. Wheat bran	7.90	"	"	"	
4. Water	0.36	"	"	litre.	

Bones.

Case No. 1	...	74.3	mg.	per	100 g.	fat-free bone.
Case No. 2	...	43.2	"	"	"	"
Case No. 3	...	37.6	"	"	"	"

Samples of milk and vegetables could not be analysed owing to putrefaction.

EXPLANATION OF PLATE V.

- Fig. 1. Rat showing moderate elongation and curvature of the upper incisors. The cutting edges are more blunt and spatulate than normally.
- „ 2. Rat showing extreme elongation and curvature of the upper and the lower incisors. Note the presence of white streaks and spots on the labial surface.

PLATE V.



Fig. 1.



Fig. 2.

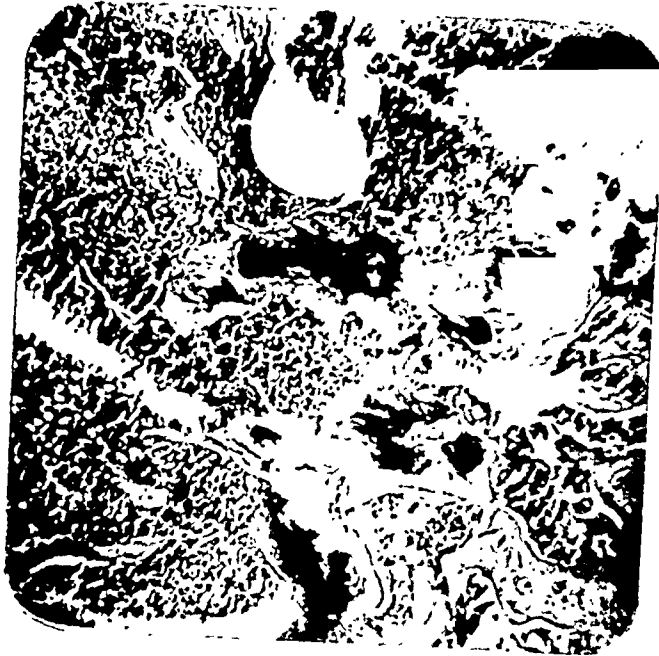


Fig. 3.



Fig. 4.



Fig. 5.

EXPLANATION OF PLATE VI.

- Fig. 3. Longitudinal section of the lower extremity of the femur showing resorption of trabeculæ, breaches in the epiphyseal line of cartilage and tongue-like projections from this line in the metaphysis undergoing ossification. $\times 250$.
- „ 4. Longitudinal section of the diaphysis of the femur. The compacta contains more osteoblasts than osteocytes, and stumpy trabeculæ arising from it show accumulations of osteoblasts at their bases. $\times 50$.
- „ 5. Longitudinal section of the femoral crest showing numerous fine dark-staining granules in the bone matrix, and around the pyknotic bone-cells. $\times 200$.

EXPLANATION OF PLATE VII.

- Fig. 6. Longitudinal section of the sternum showing thickly interwoven trabeculae and intra-trabecular spaces containing fibrous marrow. $\times 50$.
- „ 7. A ground preparation of the upper incisor tooth showing hypoplasia of the enamel organ. $\times 160$. Staining with silver nitrate.



Fig. 6.

Fig. 7.



Fig. 8.



Fig. 9.



Fig. 10.

EXPLANATION OF PLATE VIII.

- Fig. 8. A ground preparation of an upper incisor tooth. Note the presence of lighter staining and darker staining areas suggesting irregular deposition of calcium in the enamel organ. $\times 250$. Staining with silver nitrate.
- „ 9. Cross section of an upper incisor tooth showing calcification of the dentine in parallel stratified lines. Other part of this structure shows calcification in globules. Inter-globular spaces are prominent. $\times 160$.
- „ 10. A ground preparation of an upper incisor tooth showing irregular width of the pre-dentine and the wavy boundary line between dentine and pre-dentine. $\times 250$.

The softer organs showed no specific change, but the bones were brittle and chalky-white in appearance. Signs of gross abnormality were present in the columna of one case, the dorsal vertebræ showing a lateral curvature and irregular thickenings. The long bones showed no exostoses but appeared to be stouter than normal and the bony tuberosities and crests were over-prominent. The calvarium in one case of the first series was thickened and more opaque than normal owing to a chalky-white coating on its inner surface.

Histopathology.

Pieces of femur (lower end), rib, vertebræ, calvarium and sternum were decalcified in 5 per cent nitric acid and sections prepared by the usual methods.

Femur.—The epiphyseal cartilage showed a varying degree of ossification with the result that only a vestige of this line was left intact and in this the columnar arrangement of cartilage cells had disappeared. There was practically complete disappearance of the older trabeculæ in the metaphyseal region, where the cellular marrow contacts the remaining epiphyseal line. The earlier changes were apparently characterized by tongue-like projections of the epiphyseal cartilage into the metaphysis, as remnants of cartilage undergoing ossification were found in the latter region (Plate VI, fig. 3). In the main, the pathological picture was one of osteoporosis of the sub-chondral spongiosa, since resorbed trabeculæ were still to be seen in the metaphyseal region. The compacta at the level of the metaphysis and further down in the diaphysis showed irregular thickenings, giving rise at places to stumpy trabeculæ projecting into the marrow cavity (Plate VI, fig. 4). These were found studded with cells devoid of lacunar spaces, indicating thereby their recent origin. No osteoclasts were seen but newly-formed trabeculæ lined with osteoblasts were found at places. The compacta at the level of the femoral crest (Plate VI, fig. 5) showed numerous fine dark-staining granules in the lighter staining areas as well as around the pyknotic bone cells and the borders of the haversian canals. The bone-marrow was very cellular, the cells being chiefly myelocytes and well-formed erythrocytes.

Rib.—The changes observed were more or less similar to those observed in the femur.

Calvarium and sternum.—These bones were composed of thickly interwoven trabeculæ and narrow interstices, the latter containing abundant fibrous marrow (Plate VII, fig. 6).

Incisor teeth.—These tissues were decalcified in 5 per cent nitric acid and sections stained with hæmatoxylin and eosin. To study changes in the enamel, the teeth were ground very thin and stained with silver nitrate. The characteristic features were hypoplasia of the outer pigment layer of enamel organ (Plate VII, fig. 7) and deficient calcification of enamel and dentine. The outer pigment layer appeared to be irregular and broken and at places devoid of pigment, and the irregular state of calcification in the enamel rods could be easily demonstrated by silver nitrate impregnation (Plate VIII, fig. 8). The dentine, instead of being evenly calcified, appeared to be stratified in parallel lines near the pulp cavity, while further away the structure contained numerous globules and interglobular spaces (Plate VIII, fig. 9), both these features indicating defective calcification. The boundary line between pre-dentine and dentine was wavy and the zone of pre-dentine was irregular and wider than normal (Plate VIII, fig. 10).

DISCUSSION.

Three points may be discussed: (1) whether the condition here described can be attributed to any other dietary factor, (2) whether the amount of fluorine present in the food and water samples could induce pathological changes in bones and teeth, and (3) whether the osseous and the dental changes observed correspond to those of experimentally induced cases. Vitamin deficiencies though known to bring about osteoporotic changes in the metaphyses of bones in rats and guinea-pigs (Hertz, 1936) may be excluded, as the diet of our animals contained adequate quantities of vitamins. Moreover, there were no other clinical signs of avitaminoses. With regard to calcium and phosphorus, the diet fed to our rats may be considered to be

inadequate, as these being the breeding stock, no mineral supplements were given in order to meet the needs of pregnancy. Although trabecular resorption may be attributed to a certain extent to mineral imbalance, the teeth changes as described would be difficult to explain on the basis of calcium deficiency alone.

On the other hand, the amount of fluorine required to bring about characteristic changes in the teeth and bones of rats is extremely small. With a concentration between 0.0007 and 0.0014 per cent fluorine in food, Smith and Leverton (1934) were able to detect teeth changes, whereas deEds and Thomas (1933-34) found the minimum toxic dose for rats to be 6.5 to 1.0 mg. per kg. body-weight daily. From the analytical figures of fluorine in food and water of our rats, the fluorine concentration in the diet is found to be 0.0005 per cent, and the daily intake per rat 0.4 mg. per kg. body-weight. These figures are exclusive of the amount present in milk and vegetables, and as obviously some amount of fluorine was available through these two articles of diet, the rats may be presumed to have received the minimum toxic dose of the mineral. The high fluorine contents of the bones of all three cases of the first series support this hypothesis. The normal fluorine content in the bones of rats fed on a low fluorine ration (3 p.p.m. fluorine) is of the order of 15 mg./100 g. of fat-free bone, but in the three affected rats the figures varied from 37.6 mg./100 g. to 74.3 mg./100 g. of fat-free bone. From these figures it appears that the rats were receiving a diet containing approximately 8 to 12 p.p.m. of fluorine (*cf.* Ellis and Maynard, 1936), a dose which may definitely produce characteristic lesions in bone and teeth. In the pathogenesis of fluorine intoxication the rôle of calcium or the relative proportion of calcium and phosphorus in the diet has recently been emphasized by several investigators. The basal diet composed of cereal grains fed to our animals may be said to be rich in phosphorus and deficient in calcium. The calcium, available through milk and vegetables also fed to our animals may be said to have corrected the prevailing imbalance to a certain extent, but still not provided for the need of pregnancies in the case of the affected female rats. If an excess of calcium in the food resulting in positive calcium balance has a protective action against fluorine intoxication, this factor may be said to be entirely absent in respect of our rats in which 0.0005 per cent level of fluorine could be considered sufficient to precipitate the characteristic lesions and symptoms of fluorosis.

Before comparing the histopathological picture of these spontaneous cases with those observed under experimental feeding with fluorine compounds, it may be remarked that the lesions encountered by various investigators in the osseous tissues have not always been consistent. Thus, while some investigators (Bethke *et al.*, 1933; Phillips and Lamb, *loc. cit.*) found the bones of fluorine-intoxicated rats to be normal, others (Dittrich, 1932; Sutro, *loc. cit.*; Roholm, *loc. cit.*) found marked changes, varying from osteoporosis to osteosclerosis, on various levels of fluorine intake.

In histological details the natural affliction as described in this paper corresponds very closely with experimental fluorosis. The presence of irregular endosteal thickenings of the diaphyses and the fibrous replacement of marrow spaces in the calvarium and sternum are suggestive of typical osteosclerosis. The osseous changes, taken in conjunction with the gross and histological appearances in teeth as described above, are overwhelming evidence in support of the diagnosis of fluorine intoxication in these rats.

SUMMARY.

1. A condition in rats, characterized by protrusion and mottling of incisor teeth, has been diagnosed from histological examination of teeth and bones as fluorine intoxication.

2. The condition had occurred spontaneously in a colony of rats maintained on a diet from which the animals were presumably ingesting a toxic dose of fluorine. The fluorine concentration in the bones of some of the rats was much higher than that of animals kept on a low fluorine ration.

3. Histological changes in the bones and teeth of these spontaneous cases correspond closely to those of experimentally-induced cases.

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ASSAY OF ANTHRACENE PURGATIVES BY THE ESTIMATION OF THE CONTENT OF HYDROXYMETHYLANTHRAQUINONES.

Part I.

RHUBARB.

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INTRODUCTION.

RHUBARB (*Rheum*), aloe, cassia (senna) and cascara (*Rhamnus*) have been used in therapeutics as mild irritant laxatives.

As early as 1898, Tschirch and his pupils demonstrated that these aperients contain derivatives of hydroxymethylanthraquinones, either in the free state or combined as glucosides. Cruber, Bryan and Richardson (1932) have demonstrated in dogs that the anthraquinone glucosides of cascara, rhubarb and aloe produce increased peristalsis of the ileum when given intravenously, the average dose being about 5 mg. per kilo body-weight. Similar effects of parenteral administration of emodin cathartics have been reported by Dixon (1902) and others. In the usual therapeutic doses, however, all the hydroxymethylanthraquinone derivatives act mainly on the large intestine. The individual variations shown in the action of these purgatives is probably due to the chemical structure of the hydroxymethylanthraquinones, the lability of the glucosidal complex in the intestinal juice and the presence of tannins and other extractives in the drug.

The assay of these purgatives by biological methods is attended by certain difficulties. With the exception of cats, the common laboratory animals are not very suitable for the investigation of these anthraquinone purgatives (Sollman, 1942). Even in cats the method of evaluation of the purgative potency is time-consuming and can only yield qualitative values.

The British Pharmacopœia (1932) describes the botanical character of rhubarb and mentions the ash content and the alcohol (45 per cent)-soluble extractive as criteria of its purity. As regards aloe, cascara or senna, no methods are laid down for their assay and the ash and extractive serve only as guides to their purity.

The experimental difficulties about the biological assay, coupled with the fact that the hydroxymethylanthraquinones, either in the free state or in combination, are the active purgative principles of all these drugs, led us to investigate whether any correlation could be established between the content of hydroxymethylanthraquinones in these drugs and their purgative action. This would naturally lead to a method of chemical assay which would be very simple to carry out and would yield quantitative values. In the present paper, we have confined ourselves to the results on rhubarb, and we are continuing our investigation on other anthraquinone drugs available in India.

Chemical assay.—Tschirch and Edner (1907) have described a method for the estimation of the total hydroxyanthraquinones (free and combined) in rhubarb. The method is not only reliable but is simpler and quicker than those described earlier and has been followed in detail in all our estimations.

In addition to the estimation of hydroxymethylanthraquinones, an estimation of the content of tannins was carried out in order to see whether part of the variation in physiological action could be explained by variations in these. The tannin content was estimated by the copper-acetate method (Meyer's process). The moisture, ash and alcohol-soluble extractives as required by B. P. were also estimated: the results of analysis are presented in Table III.

Pharmacological assay.—Preliminary experiments with rabbits confirmed the findings of earlier workers that these animals were useless for purgation experiments; guinea-pigs were also found unsuitable. Preliminary tests proving successful, cats were finally selected for assaying the purgative potency of the anthracene purgatives. Cats between 1·5 kg. and 3·5 kg. were selected; they were kept and observed in the laboratory for 2 weeks before being used. The food, supplied once a day at 11 a.m. to these cats, was kept uniform throughout and consisted of boiled rice (90 g.), boiled fish (90 g.) and milk (60 g.). The quantity, consistency, colour and number of stools were noted very carefully every day. Usually these cats fed on laboratory diet and kept under laboratory conditions passed one slate-coloured scybalous stool about 25 g. in weight in 24 to 48 hours. The drug preparation in the required dose was suspended in about ten millilitres of tap-water, and was given by stomach tube usually at 3-30 p.m. in the afternoon. The number, quantity, consistency and colour of the stools were noted until the return to the original consistency usually within 2 to 4 days after giving the drug.

Two series of experiments were performed. In the first series (A), the animals were given an amount of alcoholic (90 per cent) extract calculated to contain 5 mg. of hydroxymethylanthraquinone, expressed as chrysophanic acid, per kg. body-weight. This extract was prepared by exhausting the powdered drug with 90 per cent alcohol and concentrating to the known strength. The calculated amount was evaporated in a water-bath and the residue suspended in about ten millilitres of water with mucilage. At least two such feeding experiments were done with each sample of extracts of different rhubarb specimens.

In the second series (B) the animals were given 3 g. of the finely-powdered drug suspended in water. Preliminary experiments with coarse powder and in 1 g. to 2 g. dose were not notably effective.

Results are presented in Tables I and II.

In Table III the relevant chemical composition of, and the pharmacological effects produced by, the different samples of rhubarb have been presented for ready comparison.

The notation (*plus* signs) is arbitrary and indicates the duration of purgative effect and fluidity of stools.

DISCUSSION.

Rhubarb and its preparations are used in medicine for two purposes. In small doses (0·05 g. to 0·3 g.) the powder is used as an astringent in gastric catarrh and diarrhoea. This astringent action is due to the tannin that all rheum preparations contain. Larger doses (1 g. to 5 g.) are used for laxative effect in occasional constipation following indigestion. Its use in habitual constipation has also been suggested though the post-purgative constipative effect should contra-indicate its use in this condition.

The dose of anthracene purgatives in cats is equal to that used in man (Sollman, *loc. cit.*). In doses less than 1 g. in the experiments reported in this paper, the different rhubarb powders produce a constipative effect. In doses varying between 1 g. and 3 g. (equivalent to 10 mg. to 30 mg. chrysophanic acid per kg. body-weight) all the rhubarb powders produce a purgative effect. As expected, the effect with the bigger dose, except in the case of European rhubarb with the highest tannin content, is more intense and prolonged. It will be clear from Table III that the samples (Sikkim L. I and L. II) containing the smallest amount of tannin produced the most prolonged action.

With the alcoholic (90 per cent) rhubarb extract which contained all the tannins, in the dose 5 mg. per kg. body-weight, the purgative effects of Sikkim G. I and G. II were the most prolonged; the tannin contents of these two samples are intermediate between those of Sikkim L. I and L. II on the one hand and European and Chinese rhubarb on the other. The purgative effect of the last two varieties (European and Chinese) containing the greatest amount of tannin is intermediate in action between those of the L. and G. varieties of Sikkim rhubarb.

TABLE I.

Experiments with alcoholic (90 per cent) extracts. Dose equivalent to 5 mg. oxymethylanthrquinone per kg. body-weight.

Weight of cat, kg.	Rhubarb extract.	Dose. Hydroxy- methylantra- quinone equivalent, mg.	CHARACTER AND COLOUR OF STOOL.				REMARKS.
			Next forenoon.	2nd day.	3rd day.	Subsequent days.	
1.5	European ..	7.5	Normal, yellow.	Soft, yellow, one motion.	Thick 'dhal' soup- like, yellow.	Soft, one on the 4th day.	Normal on the 5th day. Laxative effect +++.
1.6	Chinese ..	8	Soft, yellow.	No motion.	Thick 'dhal' soup- like.	Solid.	" " "
1.6	Sikkim L. I	8	Treacle-like, yellow.	Soft.	Solid.	"	Normal after 72 hours. Laxative effect ++.
3	Sikkim L. II	15	Semi-soft.	Semi-soft.	"	"	Normal after 48 hours. Laxative effect +.
1.56	Sikkim L. II	7.8	Treacle-like, yellow.	"	"	"	Normal after 48 hours. Laxative effect ++.
3.3	Sikkim G. I	15.15	No motion.	"	Treacle-like, yellow.	Treacle-like, yellow.	Normal after a week. Laxative effect +++.
1.98	Sikkim G. I	10	Soft, yellow.	Soft, yellow.	"	"	" " "
3	Sikkim G. II	15	Semi-soft.	Semi-soft.	"	"	" " "
2.6	Sikkim G. II	12.5	"	"	"	"	" " "

TABLE II.

Rhubarb powder. Dose 1 g. to 3 g. per kg.

			CHARACTER AND COLOUR OF STOOL.				REMARKS.	
Weight of cat, kg.	Drug.	Dose, g.	Hydroxymethyl-anthraquinone content, mg.	Next forenoon.	2nd day.	3rd day.	Subsequent days.	
3	European ..	0.65	15 (5 mg. per kg.).	Slightly soft.	No motion.	None.	None.	Normal after 5th day. Rather constipative.
3.4	European ..	1.3	30 (9 mg. per kg.).	"	Normal.	Normal.	Normal after 4 hours. Slightly or no laxative effect.
3.6	European ..	1.5	36 (10 mg. per kg.).	Soft.	"	Slight laxative effect.
1.9	European ..	3	68.4 (34.2 mg. per kg.).	"	Normal.	" "
2.5	Chinese ..	3	79.2 (31.5 mg. per kg.).	Soft, two.	Treacle-like, one.	Treacle-like, one.	None for two days.	Normal on the 6th day. Laxative effect +++.
3	Sikkim L. I	3	96 (32 mg. per kg.).	One semi-soft motion and for 5 subsequent days.	Normal after 144 hours. Laxative effect +++.
2.8	Sikkim L. II	1	27.8 (10 mg. per kg.).	No motion.	Semi-liquid, one.	Semi-liquid, one.	No motion.	Normal after 120 hours. Laxative effect +++.
3.7	Sikkim G. I	3	72 (20 mg. per kg.).	Soft, yellow.	No motion.	Semi-soft, two.	Normal.	Normal after 72 hours. Laxative effect ++.
3.7	Sikkim G. I	1.5	36 (10 mg. per kg.).	No motion.	"	No motion.	Constipative.
3	Sikkim G. II	3	59.4 (or 19.8 mg. per kg.).	Soft, yellow.	Normal.	Normal.	Laxative effect +.
2.5	Sikkim G. II	2.5	39.6 (19.8 mg. per kg.).	"	"	"	" "

TABLE III.

Source.	Moisture, per cent.	Ash, per cent.	45 per cent alcohol extractive.	Hydroxy- methyl- anthra- quinone.	Tannin, per cent.	Laxative effect 90 per cent alcohol extract 5 mg. chryso- phanic acid per kg.	Laxative effect powdered drug in 3 g. Dose 20 to 30 mg. chrysophanic acid per kg.
European	..	6.40	8.50	36.96	2.38	16.38	+++ Slight.
Chinese	..	5.36	16.00	41.60	2.64	13.50	+++
Sikkim L. I	..	5.45	10.73	43.28	3.21	9.30	++ + + +
Sikkim L. II	..	4.75	19.00	29.76	2.78	9.00	+++
Sikkim G. I	..	6.25	17.50	20.40	2.45	10.52	++++
Sikkim G. II	..	4.94	34.50	9.60	1.98	11.21	++++

Note.—B. P. limits: ash not more than 15 per cent; alcohol (45 per cent)-soluble extractive, not less than 35 per cent.

Until more is known about the enzyme lability of the hydroxymethylanthraquinone glucoside of the Sikkim L. varieties, it is difficult to explain the less marked purgative effect of these two samples containing the smallest amount of tannin. It may be due to too rapid liberation of the active anthraquinone derivative in the gut when the alcoholic extract is given, facilitating absorption into the blood stream and consequent diminution of the effect on the large intestine (Dixon, 1936). When given in bulk in powder form, the other inert and colloidal ingredients prevent this rapid liberation and consequent absorption in the upper portion of the small intestine.

The results of these experiments prove beyond doubt that the purgative effect of rhubarb in the usual cathartic dose of 1 g. to 3 g. is dependent more on their hydroxymethylanthraquinone content than on the amount of alcoholic (45 per cent) extractive present in them. The astringent effect of rhubarb is due to tannin, and all the six samples tested contained sufficient tannin to exert the binding action for which they may be used in the constipative dose. Judged by the British or the United States Pharmacopœia standards of purity of rhubarb, the Sikkim L. II, G. I and G. II would have been rejected on both counts—the ash content being too high and the extractive being too low. As has already been mentioned, pharmacologically these are, however, as good as the usual European and Chinese rhubarb. Sikkim L. I rhubarb satisfies the British Pharmacopœia standard and is also pharmacologically effective.

It is, therefore, suggested that the official standard of purity for rhubarb should be modified in the light of these findings. A hydroxymethylanthraquinone content not less than 2 per cent may be fixed as standard of purity for the present until more samples are tested.

SUMMARY.

1. The hydroxymethylanthraquinone content of rhubarb obtained from various sources [European, Chinese, Sikkim (4 samples)] has been estimated.

2. The hydroxymethylanthraquinone content of rhubarb is a correct indication of the purgative potency. The purgative effect of these different rhubarb samples have been tested on cats, and it has been found that the effect is related to the hydroxymethylanthraquinone content. Samples of rhubarb containing about 2 per cent. of hydroxymethylanthraquinone, even though they contain alcoholic extractive far below the minimum B. P. standard, are pharmacologically as effective as any B. P. standard rhubarb.

3. A modification of the present pharmacopœial standard of purity of rhubarb is suggested.

We are much indebted to Mr. J. K. Lahiri, M.Sc. (Lond.), Chemist, Indigenous Drugs Inquiry, Indian Research Fund Association, for the chemical analysis of the different samples of rhubarb.

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ON THE PREPARATION OF LIQUOR ADRENALINÆ HYDROCHLORIDI.

BY

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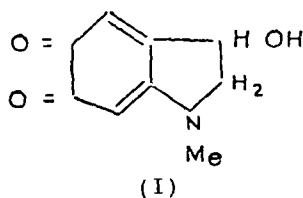
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IN the British Pharmacopœia (1932) it is recommended that adrenaline should be dissolved in an aqueous solution containing 0.9 per cent sodium chloride, 0.5 per cent chlorobutol and almost the theoretical amount of hydrochloric acid. The solution should be kept in a well-filled, well-closed container, protected from light and stored in a cool place. Our experience in this direction for the last few years tends to show that such a solution cannot maintain the usual biological activity of adrenaline as determined on a decapitated cat. On long storage such solution develops a colour and often gives rise to a precipitate. The recent researches of Richter and Blaschko (1937, *cf.* also Bergel and Morrison, 1943) tend to show that the above phenomena are due to the gradual formation of adrenochrome (I) due to the oxidation of adrenaline molecule. It has, however, been noticed that even the solution which does not contain any precipitate, nor has developed any appreciable colour, does not maintain its original potency.



The presence of a bisulphite ion in the solution has been noticed by Sjögren and Larsson (1936, *cf.* also Woolfe, 1941) to prevent the formation of a colour. Very recently, Richards (1943) has pointed out that sodium bisulphite increases the toxicity of adrenaline. Furthermore, it is a common belief that adrenaline tartrate is more stable than adrenaline hydrochloride. Considering all these points certain investigations have been carried out in this Laboratory, which are now recorded in this paper.

EXPERIMENTAL.

At first a usual liquor adrenalinae hydrochloridi was prepared and incorporated with 0.1 per cent sodium metabisulphite as this has been recommended in preparing an injection of procaine and adrenaline in the 4th Addendum to B. P., 1932. This solution was sterilized and filled into ampoules according to B. P. The ampoules used in the experiments passed the usual B. P. test for surface alkalinity of glass. These were then left aside in a dark-room and maintained at room temperature (25°C. to 30°C.). From time to time these were taken for chemical, physical and biological observations. The results obtained are recorded in Table I.

It has been previously shown by Basu, Ganguly and Bose (1944) that the preservative chlorocresol promotes the oxidation of the adrenaline molecule more than chlorobutol. The influence of sodium bisulphite has again been noticed in the course of the present work. For this adrenaline (0.1 g.) was dissolved in freshly boiled water (100 c.c.) containing molar amount of sodium bisulphite in an atmosphere of carbon dioxide and the pH was adjusted

TABLE 1.

*Effect of storage at room temperature (25°C. to 30°C.) of liquor
adrenalinae hydrochloridi with 0.1 per cent sodium
metabisulphite; pH of the solution was 2.0.*

Number of experiment.	Period of storage.	Potency by bio-assay, per cent.	Physical characteristics of the solution.
1	<i>Nil</i>	100.0	Colourless.
2	4 months	85.0	„
3	6 „	72.0	Slight pinkish.
4	8 „	65.0	Pink.
5	1½ years	48.4	„
6	3 „	44.3	Dark red with precipitate.

to *ca.* 6.5 with sulphur dioxide in one case and to 2.8 in another. The solutions were then sealed in neutral glass-ampoules. The potency of each ampoule was noticed and found to be cent per cent by the usual bio-assay method. The ampoules were then heated in a water-bath at 80°C. for 100 hours. No change in colour was noticed in these cases after heating, but the potency on biological assay on a decapitated cat was found to be practically *nil* in the former and only 45 per cent in the latter. The data recorded in Table II are from the average of three experiments:—

TABLE II.

*Change in adrenaline (0.1 per cent) solution in water with
sodium metabisulphite (0.056 per cent) when heated
at 80°C.; initial potency: 100 per cent.*

Number of experiment.	pH.	Potency.	Physical character.
1	6.5	<i>Nil</i>	Colourless.
2	2.8	45	„

To see whether any other acid can prevent the deterioration of adrenaline activity, adrenaline solution was made as usual by dissolving 0.1 g. adrenaline in 100 c.c. of water containing the molar amount of camphoric, tartaric, cinnamic and coumarin-3-carboxylic acid. Each of these solutions was then taken in a neutral glass-container and dust-free sterile air was passed through it intermittently at room temperature (25°C. to 30°C.) for a total period

of 56 hours during a week. The alterations in pH, the changes in colour, formation of any precipitate and the loss in potency are recorded in Table III :—

TABLE III.

Change in adrenaline solution prepared from organic acid.

Number of experiment.	Solution.	pH.	POTENCY.		Other change.
			Original.	After aeration.	
1	Adrenaline camphorate	5.6	100	57.1	Colour ; precipitate.
2	Adrenaline tartrate	3.9	100	86.6	„ „
3	Adrenaline cinnamate	2.8	100	80.0	Colour.
4	Adrenaline coumarin-3-carboxylate ..	5.3	100	65.0	Colour ; precipitate.

DISCUSSION.

From the data recorded in Table I it is evident that adrenaline in solution undergoes some deterioration. The observations (*vide* Table II) with bisulphite salt tend to indicate that the bisulphite ion has a direct influence in lowering the potency of adrenaline. Since sodium bisulphite has also been found to increase the toxicity of adrenaline solution (Richards, *loc. cit.*), it is a question whether any alkali metal salt of a sulphurous acid should be mixed with any preparation containing adrenaline. In British Patent 440968, a salt of adrenaline from coumarin-3-carboxylic acid has been described. In the expectation that this salt may be more resistant, this compound was prepared and its stability studied along with other organic acid (like camphoric, tartaric and cinnamic) salt of adrenaline. Table III indicates that mere incorporation of an organic acid cannot stabilize the adrenaline solution. During the course of the investigations it has always been noticed that a pH more than 3.0 and less than 1.8 tends to promote alteration in the activity of the solution more quickly.

SUMMARY.

1. Adrenaline solution as described in the British Pharmacopœia (1932) does not remain stable when incorporated with sodium bisulphite.
2. Sodium bisulphite lowers the physiological activity of the adrenaline solution. It should not be incorporated in any adrenaline preparation.
3. Mere formation of an organic acid salt of adrenaline does not ensure the stability of the solution.

In conclusion the authors wish to express their sincere thanks to Dr. U. P. Basu, D.Sc., P.R.S., for his suggestions, and to Dr. A. N. Bose, M.B., for his keen interest in the course of this investigation.

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INULA ROYLEANA DC: ITS CHEMISTRY AND PHARMACOLOGICAL ACTION.

BY

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Inula royleana is a stout perennial shrub belonging to the family *Compositæ*. It grows on the western temperate Himalayas at altitudes of 7,000 to 12,000 feet above sea-level. It is common in Kashmir and is known as 'Mallin'. In the Punjab it is called 'Mullun'. The plant is considered to be poisonous and is used by Vaid and Hakims as a disinfectant and as an insecticide, and is commonly used against the head louse. It is also used as an application in boils, inflammations, etc.

CHEMICAL COMPOSITION.

The plant does not appear to have been investigated before. For a systematic chemical examination 60 g. of the powdered root were successively extracted in a Soxhlet with different solvents as under:—

Petroleum ether 1.79 per cent, sulphuric ether 2.08 per cent, chloroform 8.44 per cent, ethyl acetate 1.17 per cent, alcohol 10.6 per cent.

The petroleum-ether extract showed the presence of a pale-yellow fixed oil and a small quantity of oleoresinous matter.

The ether extract gave further amounts of resinous matter and gave indications of the presence of traces of alkaloids.

The chloroform extract showed the presence of a fair amount of a bitter substance which gave copious precipitates with alkaloidal reagents. Ethyl-acetate extract also contained traces of a substance of alkaloidal nature and resins. The alkaloid fraction was separated from the chloroform extract and found to be soluble in nearly all organic solvents. It had a bitter taste and could not be easily crystallized.

The alcoholic extract contained some tannins and colouring matter. No substance of glucosidal nature was found to be present.

One hundred grammes of the powdered roots were steam-distilled but no essential oil was obtained.

Isolation and purification of the alkaloid.

Five pounds of the powdered drug were percolated with 90 per cent alcohol and the solvent was removed under reduced pressure. The concentrated extract was mixed with sufficient quantity of slaked lime (20 per cent of the weight of the material) and dried. The dry mass was powdered and extracted by percolation with ether in the cold. The solvent was removed, the alkaloid was dissolved in absolute alcohol and treated with animal charcoal. After concentration and allowing the solution to stand for some time, the alkaloid separated as a white crystalline substance. It melted at 120°C. to 121°C. The total quantity of alkaloid obtained amounted roughly to 3 per cent of the dried roots. After separation of

the crystals there remained behind a gummy substance which prevented further crystallization. This gummy substance also gives precipitate with some alkaloidal reagents which differ from the crystallized portion. Its physical properties and nature are under investigation.

Properties.—The alkaloid base occurred in the form of fine needle-shaped crystals when examined under the microscope (*see* Plate IX). It has a very bitter taste. It is soluble in nearly all organic solvents except petroleum ether and gives a faint-violet fluorescence. It is very slightly soluble in water to which it imparts a faint bitter taste. It gives precipitate with all the alkaloidal reagents, but no colour reaction with mineral acids. It crystallizes readily from ether and alcoholic solution, but not so from other solvents. It has distinctly basic properties and forms salts with mineral acids but attempts to crystallize these failed. The only crystalline salt it formed was the gold salt, which occurred as beautiful leafy stellate crystals. The specific rotation of the alkaloid in absolute alcohol solution is $(L) 17_D - 42.5$.

The proximate analysis of the alkaloid gave—

			Per cent
C	62.17
H	9.45
O	25.23
N	3.51

from which the empirical formula was found to be $C_{21} H_{38} O_6 N - 400$.

Gold salt.—This was prepared by adding a two per cent solution of gold chloride to a solution of the alkaloid in dilute hydrochloric acid, when the gold salt is precipitated as a yellow amorphous powder. The amorphous salt was dissolved and crystallized from hot water: it melts at $142^\circ C$. with decomposition. Determination of molecular weight is being carried on. The alkaloid is provisionally named 'Roylene'.

Pharmacological action.

Action on paramœcia.—The action of the plant on paramœcia was tested with (a) a solution of the hydrochloride of the purified alkaloid and (b) decoction of the dried roots of the plant. It was found that the alkaloid in concentrations such as 1 in 400 kills paramœcia in 27 minutes and 1 in 200 takes 12 minutes to kill all the paramœcia. The decoction of the root of the plant is, however, comparatively much weaker, so much so that a dilution of 1 in 500 kills only about 50 per cent paramœcia in about an hour's time. The alkaloid is thus not very toxic to these ciliates.

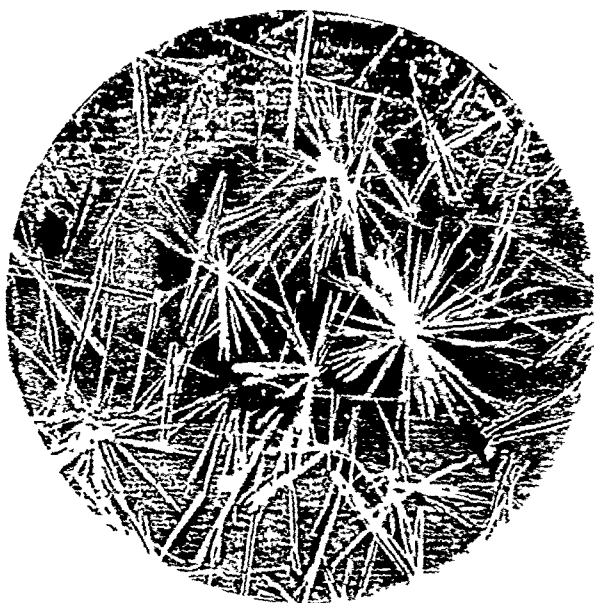
Action on bacteria.—One per cent solution of the alkaloid was mixed with an equal quantity of 18-hour-old broth cultures of *E. typhi* (T.E.D. strain). At intervals of fifteen minutes, loopfuls of this mixture were inoculated into nutrient broth (5 c.c.; pH 7.6) for a total period of three hours. All the tubes thus inoculated were incubated at $37^\circ C$., and examined for growth after 24 hours. All tubes showed growth. The alkaloid, therefore, has no bactericidal action.

Local action.—When applied locally to intact skin, the alkaloid does not produce any signs of irritation. It also does not produce any congestion of the conjunctiva or any effect on pupil when instilled into the eye.

Effect on blood-pressure.—In intact urethanized cats the alkaloid administered intravenously in doses varying from 2 mg. to 5 mg. per kg. body-weight produces a sudden fall in the blood-pressure ranging from 10 mm. to 40 mm. The blood-pressure, however, gradually rises for 8 to 10 minutes but does not attain the original level. This effect of the alkaloid progressively diminishes after the first one or two doses (dose per dose). In spinal animals 2 mg. of the alkaloid does not produce any change.

Effect on respiration.—The effect of the alkaloid on respiration was tested by observing the intratracheal pressure in cats under urethane, and it was found that in doses from 2 mg. to 5 mg. per kg. it has no effect on respiration.

PLATE IX.



Crystals of alkaloid 'Roylene' from absolute alcohol.

GLYCOLYSIS IN DIABETIC BLOOD AND ITS SIGNIFICANCE.

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INTRODUCTION.

In a previous paper (Bose and De, 1942) the effects of temperature and anticoagulants on glycolysis in normal blood were studied and the main findings arrived at were that the sugar content of blood undergoes gradual loss *in vitro* and that the rate of glycolysis depends on the temperature at which the samples are kept and the anticoagulants that are used.

During the course of the above work on glycolysis of normal blood, we made a few casual observations on diabetic blood as well and it appeared to us that the rate of glycolysis in the latter cases differed materially from that of the former, sometimes in a very marked degree. From the literature it also appeared to us that opinions were not uniform with regard to the question of the glycolytic rate in diabetic blood. Lepine (1909), for instance, showed that the rate of glycolysis in diabetic blood was much lower than normal. Denis and Giles (1923) reported that the glycolytic power in the blood of diabetic patients was less than normal. Thalhimer and Perry (1923) also showed that the glycolysis proceeded at a slower rate in diabetic blood samples. Tolstoi (1924), on the other hand, did not observe any difference in the glycolytic power of diabetic blood as compared to normal. Cajori and Crouter (1924) also supported Tolstoi in the observation that there was no diminution of the glycolytic power in the diabetic blood as compared to that of normal.

These conflicting opinions led us to investigate the following points :—

1. Does the glycolytic power of diabetic blood differ materially from that of the non-diabetic ?
2. Does the rate of glycolysis in diabetic blood samples depend in any way on the severity of the diabetic condition ?
3. Does the rate of glycolysis alter when the diabetic condition improves as a result of treatment ?

Preliminary observations were made on 30 serial cases of diabetes irrespective of severity. The rate of glycolysis was studied in this group of cases hourly for 6 hours. All the blood samples were collected in 'fasting' condition. Potassium oxalate was used as an anticoagulant and the samples were all kept at room temperature (23°C. to 29°C.). The subsequent treatment of the blood samples was exactly the same as described previously (Bose and De, *loc. cit.*).

The results obtained from the 30 cases examined are summarized below in tabular form :—

Number of cases.				Total loss of sugar at the end of 6 hours (in mg. per cent).
In 12 cases	56 to 58
" 7 "	15 to 21
" 9 "	7 to 17
" 2 "	Nil.

TABLE I.

Mild cases.

Sub-group.		AVERAGE BLOOD-SUGAR VALUES.										AVERAGE LOSS OF SUGAR IN MG. PER 100 C.C.								Average total loss of sugar at the end of 6 hours.		Average total loss of sugar at the end of 24 hours.	
		Initial.	1 hour after.	2 hours after.	3 hours after.	4 hours after.	5 hours after.	6 hours after.	24 hours after.	1st hour.	2nd hour.	3rd hour.	4th hour.	5th hour.	6th hour.	24th hour.							
1	151.4	143.9	134.1	124.2	114.4	107.0	98.8	78.2	7.5	9.8	9.9	9.8	7.4	8.2	20.6	52.6	73.2						
2	158.2	150.9	143.4	133.8	123.0	113.8	104.3	89.1	7.3	7.5	9.6	10.8	9.2	9.5	15.2	53.9	69.1						
3	156.8	148.6	141.4	133.2	124.8	116.3	107.1	87.0	8.2	7.2	8.2	8.4	8.5	9.2	20.1	49.7	69.8						
TOTAL AVERAGE.		155.4	147.8	139.6	130.4	120.7	103.4	84.7	7.7	8.2	9.2	9.7	8.3	9.0	18.6	52.1	70.7						

TABLE II.

Moderate cases.

Sub-group.		AVERAGE BLOOD-SUGAR VALUES.								AVERAGE LOSS OF SUGAR IN MG. PER 100 C.C.							Average total loss of sugar at the end of 6 hours.		Average total loss of sugar at the end of 24 hours.	
		Initial.	1 hour after.	2 hours after.	3 hours after.	4 hours after.	5 hours after.	6 hours after.	24 hours after.	1st. hour.	2nd hour.	3rd hour.	4th hour.	5th hour.	6th hour.	24th hour.				
1	189.4	185.9	181.8	178.6	176.5	174.6	172.6	162.8	3.5	4.1	3.2	2.1	1.9	2.0	9.8	16.8	26.6			
2	198.2	194.1	190.0	186.2	183.4	180.4	178.3	169.7	4.1	4.1	3.8	2.8	3.0	2.1	11.6	19.9	31.5			
3	196.4	192.6	189.6	186.6	184.0	182.0	180.2	170.0	3.8	3.0	3.0	2.6	2.0	1.8	10.2	16.2	26.4			
TOTAL AVERAGE.		194.6	190.8	187.1	183.8	181.3	179.0	177.1	3.8	3.7	3.3	2.5	2.3	2.0	10.6	17.6	28.2			

TABLE III.

Severe cases.

Sub-group.	Initial.	AVERAGE BLOOD-SUGAR VALUES.						AVERAGE LOSS OF SUGAR IN MG. PER 100 C.C.						Average total loss of sugar at the end of 6 hours.	Average total loss of sugar at the end of 24 hours.
		1 hour after.	2 hours after.	3 hours after.	4 hours after.	5 hours after.	6 hours after.	24 hours after.	1st hour.	2nd hour.	3rd hour.	4th hour.	5th hour.	6th hour.	24th hour.
1	572.1	571.3	571.2	569.8	568.6	568.0	567.2	562.1	0.8	0.1	1.4	1.2	0.6	0.8	5.1
2	389.5	388.1	386.4	385.3	383.4	381.8	381.0	374.8	1.4	1.7	1.1	1.9	1.6	0.8	6.2
3	420.9	419.4	417.9	416.4	415.0	413.3	411.5	405.5	1.5	1.5	1.5	1.4	1.7	1.8	6.0
Total AVERAGE.	460.8	459.6	458.5	457.1	455.6	454.3	453.2	447.4	1.2	1.1	1.3	1.5	1.3	1.1	5.7
															13.3

These observations show clearly that the rate of glycolysis is not uniform in all cases of diabetes. Twelve of the cases examined behaved in a more or less normal manner (56 to 58 per cent loss), while in others the rate of glycolysis was reduced by variable degrees, the most striking being the 2 cases which showed no glycolysis at all. This led us to suspect that the severity of the diabetic condition might influence the rate of glycolysis.

A more critical investigation was therefore undertaken, in which the cases were grouped as mild, moderate or severe. Thirty cases of diabetes were studied in each of the 3 groups, the rate of glycolysis being determined by observations made hourly for 6 hours and again at the end of 24 hours.

For convenience and brevity of records, the 30 cases in each group have been further sub-divided into 3 sub-groups each of 10 cases and the average results of each of these sub-groups have been given in tabular form. Table I summarizes the results obtained from mild cases of diabetes, Table II from moderately severe cases and Table III from very severe cases.

An analysis of the results contained in Tables I, II and III shows that:—

In mild cases (Table I).—

1. The average hourly loss of sugar during the first 6 hours varied from a minimum 7.7 mg. to a maximum 9.7 mg. per cent.
2. The average *total* loss of sugar at the end of 6 hours and 24 hours was 52.1 mg. and 70.7 mg. per cent respectively.

In moderately severe cases (Table II).—

1. The average hourly loss of sugar during the first 6 hours varies from a minimum 2.0 mg. to a maximum 3.8 mg. per cent.
2. The average *total* loss at the end of 6 and 24 hours was 17.6 mg. and 28.2 mg. per cent respectively.

In severe cases (Table III).—

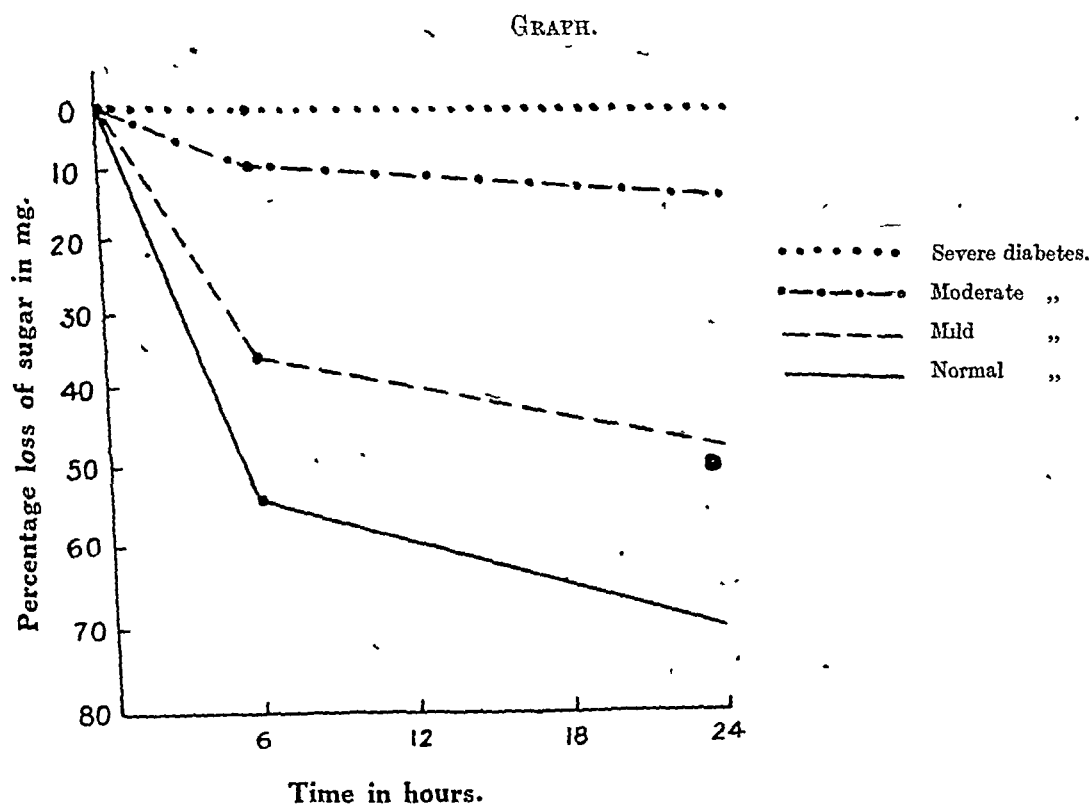
1. The average hourly loss of sugar during the first 6 hours varies from a minimum of 1.1 mg. to a maximum of 1.5 mg. per cent.
2. The average *total* loss at the end of 6 and 24 hours was 7.6 mg. and 13.3 mg. per cent respectively.

The results of this analysis are concisely summarized in Table IV. Estimations on normal cases (as published in our previous paper) are included for comparison.

TABLE IV.

		Average maximum hourly loss of sugar in mg. per 100 c.c.	Average total loss at the end of 6 hours in mg. per 100 c.c.	Average total loss at the end of 24 hours in mg. per 100 c.c.
Mild	..	9.7	52.1	70.7
Moderate	..	3.8	17.6	28.2
Severe	..	1.5	7.6	13.3
Normal cases	..	12.6	53.5	71.1

The percentage loss of sugar in mild, moderate and severe cases of diabetes, as estimated at 6 and 24 hours, is shown in the Graph in comparison with the loss in normal individuals :—



The data given above show that the rate of glycolysis is not uniform in cases of diabetes and that it varies according to the severity of the diabetic condition. In mild cases of diabetes the rate of loss of sugar per hour, as also the total loss of sugar at the end of 6 and 24 hours, does not differ much from that observed in non-diabetic cases (Bose and De, *loc. cit.*).

In moderately severe cases of diabetes, however, the rate of hourly loss of sugar, as well as the total loss at the end of 6 and 24 hours, is definitely slower than in mild cases.

In severe cases of diabetes the hourly loss of sugar during the first 6 hours is negligible and in some cases there is no loss at all. The total loss at the end of 6 hours, as well as at the end of 24 hours, is strikingly less than in mild and moderate cases.

The glycolytic rate in diabetic blood is, therefore, in inverse proportion to the severity of the diabetic conditions.

Effect of treatment on the glycolytic rate in diabetes.

The effect of intensive treatment of the diabetic condition on the glycolytic rate was studied in 10 cases of fairly severe diabetes. The glycolytic rate of the blood in these patients was estimated before and after treatment with insulin and the results are given in Tables V, VI and VII.

Table V records the glycolytic rate in the 10 selected cases before treatment was commenced.

Table VI records the results obtained in the 7 cases in which a satisfactory response to treatment was observed.

Table VII records the results in the 3 remaining cases in which response to treatment was unsatisfactory.

TABLE V.

Rate of glycolysis in 10 cases of severe diabetes before treatment.

BLOOD SUGAR MG. PER CENT.			LOSS OF SUGAR MG. PER CENT PER HOUR.						Total loss of sugar.
Case number.	Initial.	At the end of 6 hours.	1 hour.	2 hours.	3 hours.	4 hours.	5 hours.	6 hours.	
1	410	404	2	1	1	2	6
2	236	231	..	1	1	1	1	1	5
3	320	312	..	2	2	2	2	..	8
4	300	286	4	1	3	3	2	1	14
5	410	403	..	2	1	..	2	2	7
6	315	301	2	3	2	2	2	3	14
7	410	395	3	2	3	2	3	2	15
8	500	497	2	1	3
9	263	247	4	2	2	3	2	3	16
10	370	365	..	2	2	1	5
AVERAGE	353.4	344.1	1.5	1.6	1.6	1.4	1.7	1.5	9.3

TABLE VI.

Rate of glycolysis in 7 cases of severe diabetes under insulin treatment to which the patients responded satisfactorily.

BLOOD SUGAR MG. PER CENT.			LOSS OF SUGAR MG. PER CENT PER HOUR.						Total loss of sugar.
Case number.	Initial.	At the end of 6 hours.	1 hour.	2 hours.	3 hours.	4 hours.	5 hours.	6 hours.	
1	127	84	7	7	7	6	7	9	43
2	110	62	10	7	9	7	8	7	48
3	100	49	8	7	9	11	9	7	51
4	108	58	8	8	9	11	7	7	50
6	115	70	9	6	7	8	7	8	45
7	125	83	8	6	7	8	6	7	42
9	98	54	8	7	5	9	8	7	44
AVERAGE	111.9	65.9	8.3	6.8	7.5	8.5	7.4	7.4	46.1

TABLE VII.

Rate of glycolysis in 3 cases of severe diabetes under insulin treatment to which the patients responded unsatisfactorily.

BLOOD SUGAR MG. PER CENT.			LOSS OF SUGAR MG. PER CENT PER HOUR.						Total loss of sugar.
Case number.	Initial.	At the end of 6 hours.	1 hour.	2 hours.	3 hours.	4 hours.	5 hours.	6 hours.	
5	195	170	5	4	4	4	3	5	25
8	200	178	4	4	2	3	5	4	22
10	198	175	4	3	4	5	4	3	23
AVERAGE	197	174.3	4.3	3.6	3.3	4.0	4.0	4.0	23.3

The results recorded in Tables V, VI and VII are summarized in Table VIII in which the results formerly obtained (Bose and De. *loc. cit.*) in normal subjects have been included for comparison.

TABLE VIII.

Glycolysis in cases of severe diabetes before and after treatment as compared with the normal subjects.

	BEFORE TREATMENT.	AFTER TREATMENT.		Average results obtained in normal subjects (Bose and De, <i>loc. cit.</i>).	Average results obtained in cases of severe diabetes.
	Average results obtained in 10 severe cases of diabetes.	7 cases in whom the response to treatment was satisfactory.	3 cases in whom the response to treatment was unsatisfactory.		
Average maximum hourly loss of sugar mg. per 100 c.c.	1.7	8.5	4.3	12.6	1.5
Average total loss of sugar at the end of 6 hours (in mg. per 100 c.c.).	9.3	46.1	23.3	53.5	7.6

It is apparent from these observations that :—

1. In untreated cases of severe diabetes glycolysis proceeds at an extremely slow (almost insignificant) rate.
2. In cases which respond satisfactorily to treatment there is a simultaneous marked improvement in the rate of glycolysis.
3. In cases in which the response to treatment was unsatisfactory the glycolytic rate shows little improvement.

DISCUSSION.

The cause of the marked difference which has been demonstrated between the glycolytic rate of the blood of diabetic and the non-diabetic subjects and also the reason for the progressively diminishing glycolytic power of the blood of diabetic subjects according to the severity of the diabetic condition has not yet been determined.

The glycolytic rate of the blood, according to our observations, bears no relationship to the height of the blood-sugar level but appears to depend on other factors, possibly on a change in the chemical nature of the glucose present in the blood.

The nature of the glucose present in the circulating blood has been and still is a subject of active study, no definite conclusions having been arrived at yet. The consensus of opinion amongst most physiologists is that the form of glucose which is readily fermentable or easily assimilable in the animal organism has a ring structure of γ glucoside.

Normally, glycolysis is caused through oxidizing ferments, of which oxidases are examples. These oxidases, with the help of the insulin in the circulating blood, probably effect an alteration in the chemical structure of the glucose and makes it readily available to the tissues for its final breakdown and utilization.

Insulin can thus be assumed to be the controlling factor of these oxidizing ferments which are stimulated or activated by its action. The retardation of glycolysis in diabetes, which we know is caused through hypo-insulinism (in varying degree), may thus be partially explained.

It should be noted, however, that when insulin is added to the blood *in vitro* no alteration in the glycolytic rate is found to take place. Intensive insulin treatment, however, causes a marked increase in the glycolytic power of the blood of diabetic patients. It is, therefore, safe to assume that insulin helps to exert some indirect stimulating or activating action on the oxidizing ferment mechanism within the body which possibly causes changes in the nature of glucose in the blood of the diabetic patients converting it to a fermentable product which is capable of glycolysis.

SUMMARY AND CONCLUSIONS.

1. The rate of glycolysis in diabetic patients has been found to vary according to the severity of the condition.
2. In severe cases of diabetes there is practically no glycolysis, even if the blood samples are left at room temperature for 24 hours.
3. In moderately severe cases of diabetes glycolysis proceeds at a very slow rate.
4. The rate of glycolysis in the blood of mild cases of diabetes does not differ materially from that observed in normal persons.
5. The glycolytic power of the blood of diabetic subjects who respond satisfactorily to specific treatment improves *pari passu* with improvement in the clinical condition of the patient, and in some cases, approaches that observed in normal subjects.
6. The height of the blood-sugar level has been found to bear no relationship to the rate of glycolysis.

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ON THE ASSAY OF UREA STIBAMINE.

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INTRODUCTION.

UREA STIBAMINE is a pentavalent antimony compound first synthesized by Brahmachari in 1922, and extensively used in India in the treatment of kala-azar. Ghosh and Chatterjee (1928), Gray and Trevan (1931) and Guha, Dutt and Mukerji (1943) have all reported variations in the content of total antimony and urea in different batches of urea stibamine. Their findings support the view that, in spite of the established therapeutic efficacy of the drug in kala-azar, urea stibamine is not a compound the composition of which conforms to a uniform formula of known chemical structure.

It is obviously necessary to establish accurate control of the toxicity of the commercial preparations of urea stibamine issued for clinical use. No standard for organic antimony compounds has yet been evolved nor are there any recognized criteria by which toxicity of various samples can be assayed. Attempts by Napier (1928) to make good these deficiencies were made on an insufficiently large number of animals, while the work of Gray *et al.* (*loc. cit.*) was concerned only with investigations on the active component of antimony compounds. The absence of a dose-response curve to facilitate the assay of urea stibamine has been keenly felt in this Laboratory. For this reason, it was decided to select a batch of urea stibamine which would serve as a temporary standard and to work out in detail the chemical specifications and toxicity levels of this selected batch. The results of this work are recorded below.

MATERIALS AND METHODS EMPLOYED.

Batch No. 0596 of urea stibamine manufactured at the Brahmachari Institute, Calcutta, was selected. Ten grammes were obtained in glass-ampoules and stored at 0°C. This sample was found to have the following chemical composition:—

Physical characters—greyish-white amorphous powder.

Solubility—readily soluble in water.

Chemical content—total antimony 40.76 per cent.

Antimonious acid 2.7 per cent (expressed as antimony).

Antimonic acid—present.

Total antimony was determined by the method described by Ghosh *et al.* (*loc. cit.*). Antimonious acid was determined by directly titrating the urea stibamine with N/100 iodine in the presence of excess of sodium bicarbonate.

Animal experiments were carried out exclusively on white mice from the stock of the Central Research Institute, Kasauli. As mice of 13 g. to 15 g. weight were not available, animals weighing between 18 g. and 20 g. with a sex ratio of 2 male to 1 female for each dosage were used. The dosage per mouse was calculated on the basis of 19 g. weight and the group weight of mice for each dosage was kept constant. The injections were given intravenously at the rate of 0.01 c.c./sec. and the total mortality figures were recorded within 72 hours of injection. Other precautions recommended for the study of toxicity of the arsenicals were also strictly followed.

Solutions for injection were freshly prepared in sterile re-distilled water freed from CO₂ and sealed with a layer of sterile paraffin following the same precautions as for arsenicals. All injections were given within 15 minutes of the preparation of the solution. As the volume

injected was kept constant at 0.2 c.c. per mouse, the concentration of solutions for dosages between LD₀ and LD₁₀₀ varied from 0.95 to 3.3 per cent. The pH of the solutions in these concentrations varied between 6.5 and 6.3.

DOSE-RESPONSE CURVE.

LD₅₀ and toxicity levels estimated by Kärber's formula.—As this method gives dependable and accurate results with a minimum number of animals it was selected for preliminary study of toxicity to determine the approximate range of dosages on which to work out the dose-response curve. A series of 9 doses were selected and for each dose an equal number of male and female mice of equivalent weight were used. The injections were given within a period of 3 days.

TABLE I.
Showing dose-mortality figures in Kärber's formula.

Log dose.	Log equivalent, mg./kg.	Observed mortality within 72 hours of injection.
2.10	125.9	0/6
2.15	141.3	1/6
2.20	158.5	3/6
2.25	177.8	3/6
2.30	199.5	1/6
2.35	123.9	1/6
2.40	251.2	4/6
2.45	281.8	4/6
2.50	316.2	6/6

On the basis of the above findings LD₅₀ was worked out on the following formula:—

$\text{Log LD}_{50} = X_0 - \sum \frac{(p_1 + p_2)d}{2}$, where $X_0 = 2.50$. The sum of the 7 products obtained from the 9 dosages in this series = 0.1625.

Therefore, $\text{log LD}_{50} = 2.50 - 0.1625 = 2.5 - 0.1625 = 2.3375$

LD₅₀ = 217 mg./kg.

Dose-mortality curve.—The dose-mortality curve was worked out using 7 dosages on the arithmetic scale in 320 mice. The result is given in Table II:—

TABLE II.
Mortality figures obtained with 7 dosages of urea stibamine.

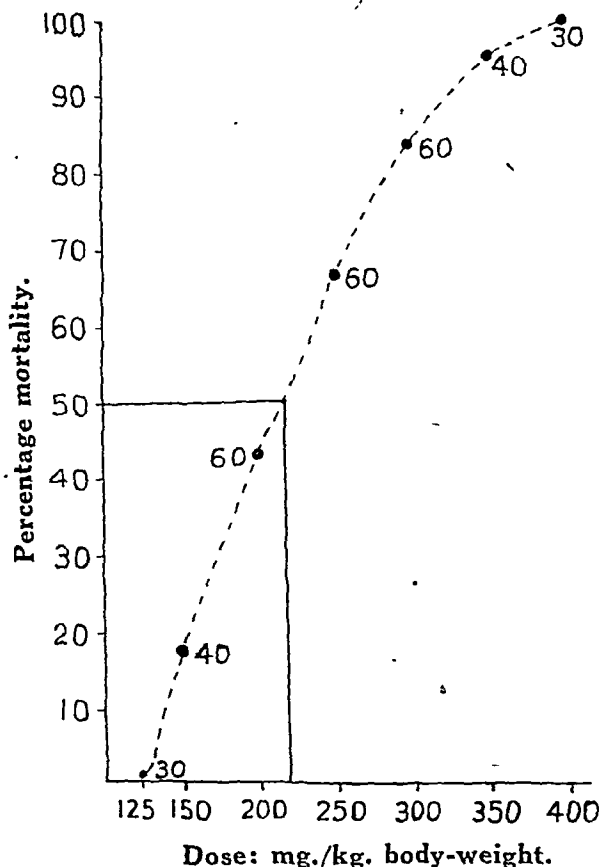
Dose, mg./kg.	OBSERVED MORTALITY WITHIN 72 HOURS OF INJECTION.						Total mortality.	Percentage mortality.
	Series No. 1.	Series No. 2.	Series No. 3.	Series No. 4.	Series No. 5.	Series No. 6.		
125	0/10	0/10	0/10	0/30	0.0
150	1/10	2/10	1/10	3/10	7/40	17.5
200	5/10	2/10	6/10	4/10	5/10	4/10	26/60	43.3
250	8/10	4/10	7/10	6/10	7/10	8/10	40/60	66.6
300	7/10	8/10	8/10	9/10	8/10	10/10	50/60	83.3
350	9/10	10/10	9/10	10/10	38/40	95.0
400	10/10	10/10	10/10	30/30	100.0

It is evident that a dose of 125 mg./kg. weight of animal represents LD₀ for 30 mice. This dose is much lower than what has been reported by Napier (*loc. cit.*), and by Guha *et al.* (*loc. cit.*). A dose of 400 mg./kg. weight for a similar number of mice represents LD₁₀₀. This is higher than what has been obtained with 6 mice in Table I with Kärber's formula.

The findings in Table II are represented in Graphs 1 and 2 :—

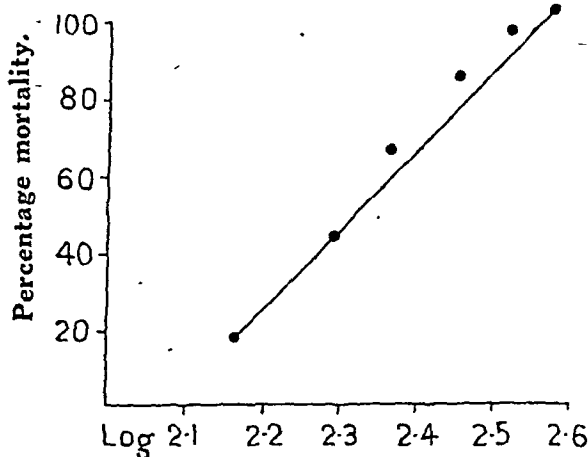
GRAPH 1.

Dose-mortality curve with doses in mg./kg. weight as abscissæ and percentage mortality as ordinates.



GRAPH 2.

Dose-mortality curve with corresponding log doses as abscissæ and percentage mortality as ordinates.



Graph 1 is a typical sigmoid curve with no skew or exponential characteristics. The upper and lower tails do not show much lag.

Graph 2 is linear in distribution and this is in conformity with the characteristics of all log curves. The LD₅₀ dose for urea stibamine is 212 mg./kg. weight of mice. This is very close to the figure of 217 mg./kg. obtained by us using Kärber's formula, and is significantly different from the figure of 250 mg./kg. obtained by Napier (*loc. cit.*). Though the percentage increase in response for doses in the region of LD₅₀ is greater than at the region of upper and lower doses it is not as great as in the case of the arsenicals. The significance of this point is described below :—

Comparison of urea stibamine and neoarsphenamine response.—The mortality due to various doses of urea stibamine can be compared with the corresponding figures in the literature for arsenicals when the doses are expressed as relative percentage of LD₅₀ taking the latter as 100. For 18 g. to 20 g. mice the toxicity of urea stibamine is higher than arsenicals when the LD₅₀ figures only are taken into consideration. Urea stibamine—212 mg./kg. (present

authors); neoarsphenamine—370 mg./kg. (Taylor and Ahuja, 1935), 470 mg./kg. (Durham *et al.*, 1929).

It is, however, very interesting to compare the relative sensitiveness in the response of the two classes of organo-metallic compounds. This has been done in Table III. A slight increase in the dose of the arsenicals above the LD₅₀ results in a much higher mortality than in the case of urea stibamine, when the dose of the latter also is correspondingly increased. Similarly, when the dose is reduced below LD₅₀ the reduction in mortality is greater in the case of arsenicals than in urea stibamine. Slight differences in dosages are more markedly reflected in the mortality figures in the case of arsenicals than in the case of urea stibamine. It thus appears that although the toxicity of urea stibamine is higher than the arsenicals as judged by their respective LD₅₀ figures, the margin that should be allowed in passing samples of urea stibamine can be greater than for the arsenicals. Comparative data on this point are given in Table III:—

TABLE III.
Showing relative distribution of toxicity figures of urea stibamine and neoarsphenamine when LD₅₀ is given an arbitrary value of 100.

UREA STIBAMINE		NEOARSPHENAMINE.	
Relative dose, per cent.	Percentage mortality.	Relative dose, per cent.	Percentage mortality.
64	6	64	6
71	18	80	18
83	30	90	30
100	50	100	50
137	80	113	80
165	95	136	95
190	100	155	100

Figures deduced from the dose-response curve of Durham, Gaddum and Marchal (1929).

Toxicity in relation to sex of mice.—In a series of 350 mice, of which there were 240 males and 110 females, 136 and 56 mice died respectively representing a mortality of 56 per cent for male and 51 per cent for female mice. This finding is interesting in as much as Durham, Gaddum and Marchal (1929) observed that, with arsenicals, female mice of 18 g. to 20 g. were more susceptible to the toxic effects than males.

Stability of urea stibamine solutions.—In the experiments described in this paper the same elaborate precautions were observed in the preparation and use of solutions of urea stibamine as are recommended in the assay of organic arsenicals. This is apparently unnecessary as is shown by the following observations:—

TABLE IV.

Nature of solution.	Toxicity within 10 minutes of preparation of solution.	Percentage mortality.	Toxicity—7 days after preparation of solution.	Percentage mortality.
Sealed with paraffin and stored at 0°C.	3/8+4/7	46.6	5/8+3/7	53.3
Unsealed and stored at 37°C.	4/8+4/7	53.3	3/8+3/7	40.0

Morrell and Chapman (1933) and Wien (1935, 1936) observed greatly increased toxicity in arsenic solutions prepared without the special precautions recommended (an increase in toxicity by 56 per cent and 17.5 per cent after 25 minutes and 15 minutes of the preparation of the solution). The findings in Table IV though collected from a small group of animals show that urea stibamine solution is much more stable than that of arsenic and such rigid precautions as are observed for working with arsenic solutions may not be necessary for urea stibamine.

DISCUSSION.

Gray *et al.* (*loc. cit.*) considered urea stibamine to be a complicated mixture of colloids and not a definite chemical compound. Of the various compounds that may be formed by the combination of urea and p-amino phenyl stibinic acid, the di-substituted urea s-diphenyl carbamide—4 : 4'—di-stibonic acid was found by Gray to be the protozoocidal constituent of urea stibamine.

Ghosh *et al.* (*loc. cit.*) reported a wide variation of antimony and free-urea content of different batches of urea stibamine. More recent workers, Gray *et al.* (*loc. cit.*) and Guha *et al.* (*loc. cit.*), have observed a smaller but nevertheless significant variation in the total antimony content of market samples of urea stibamine. The exact correlation between antimony content, toxicity and therapeutic efficiency of urea stibamine is not known. The establishment of such a correlation is not easy as urea stibamine is not a definite chemical compound. The assay of urea stibamine samples by any chemical method alone cannot, therefore, be considered satisfactory in our present state of knowledge. Reliance will, therefore, have to be placed on the biological control of these drugs until chemically pure compounds of uniform composition are available in the market.

The essential prerequisites for accurate bio-assay of any drug are the establishment of a dose-response curve with a known sample of that preparation. The chemical specification and toxicity levels of an arbitrary sample of urea stibamine have, therefore, been studied to establish a temporary standard with which to compare commercial samples. By employing two different methods of LD₅₀ determination the figures of 217 mg./kg. and 212 mg./kg. have been obtained. For convenience of calculation the LD₅₀ figure could be fixed at 215 mg./kg. mice of 18 g. to 20 g. body-weight. The distribution of the dose-response curve of urea stibamine when compared with that of neoarsphenamine brings out several points which should be taken into consideration in the assay of urea stibamine samples. The LD₅₀ figure for urea stibamine is very much lower than that of neoarsphenamine (0.21 mg. instead of 0.47 mg. per gramme weight of mice). The former is, therefore, more toxic than the latter. But the discriminating power of close doses of urea stibamine in the region of LD₅₀ is less than that of the arsenicals. It would, therefore, appear that a wider margin of variation could be permitted to urea stibamine than is done for the arsenic compounds. An allowable margin of ± 20 per cent permissible to the acceptance of the arsenicals roughly corresponds with ± 30 per cent of urea stibamine from the standpoint of toxicity. This may be considered to be the maximum permissible limit for the acceptance of unknown commercial samples of urea stibamine. The authors have also worked on a smaller margin of ± 15 per cent (± 30 mg./kg. of LD₅₀) in a number of cases. If during further assay of samples this limit is not found to be too severe, this range of 189 mg./kg. and 245 mg./kg. would be suitable for working out the median lethal dose of samples ensuring at the same time the therapeutic efficiency of the dose at its optimum level.

Another important point is that in view of the indefinite composition of urea stibamine it would be advisable to determine the LD₅₀ of every batch of this preparation by the method of bracketing with a lower and higher dose on groups of 30 mice and then working out the LD₅₀ from these results. The acceptance of samples from results obtained at the lower level alone on groups of 10 mice as is done for the arsenicals is considered risky as some non-toxic and inactive preparations could easily pass this test. This would not occur if the other method is employed.

SUMMARY AND CONCLUSIONS.

1. A selected batch of commercial urea stibamine has been adopted as a temporary standard for the assay of unknown preparations of urea stibamine. The chemical composition

of the selected batch has been determined and a dose-response curve has been worked out in white mice.

2. The LD₅₀ figure for the selected batch of urea stibamine was found to be 215 mg./kg. for white mice of 18 g. to 20 g. weight. It is suggested that in the assay of unknown samples of urea stibamine a maximum variation of ± 30 per cent on this figure should be permitted. No significant difference in susceptibility was observed in male and female mice within the limit of weight stated above.

3. Solutions of urea stibamine are much more stable than solutions of organic arsenic compounds and it is not considered necessary in assaying urea stibamine to observe the elaborate precautions in the preparation and use of solutions which must be followed in the case of organic arsenicals.

4. The relationship between the total antimony content of urea stibamine and its toxicity is not yet known. As the chemical composition of urea stibamine is variable and as it is not yet definitely known which fraction or fractions are therapeutically active, the assay of unknown preparations of this drug should, for the present, be carried out by biological methods.

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CHEMISTRY AND PHARMACOLOGY OF SOME COMMON INDIGENOUS REMEDIES.

BY

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THE present paper embodies the results of the studies carried out regarding the chemical composition and pharmacological action of some remedies widely used in indigenous medicine. Although these plants are reputed to possess certain medicinal properties and are quite commonly employed for such purposes, our observations show that, apart from the action of some known chemical substances which happen to be a part of the constituents of such plants, there is in the case of some plants an insufficient quantity of the active principles to produce any marked physiological action, and in case of others, the principle itself is inert and inactive. The following plants were studied :—

I. *Aconitum heterophyllum* Wall.

This plant is widely used in indigenous medicine and is reputed to possess antipyretic and antiperiodic properties. It is also considered to be an astringent, stomachic and aphrodisiac. It is a non-poisonous Indian aconite and grows in nature in the western temperate Himalayas from Kumaon to Hasora at altitudes of 8,000 to 15,000 feet above sea-level.

Chemistry.—The active principle of the plant is a non-poisonous alkaloid called atisine. It was isolated by Jowett (1896) who studied it and gave the formula for atisine as $C_{22}H_{31}O_2N$. Recently, Lawson and Topps (1937) prepared this alkaloid in purer form by a much simpler method and modified the formula to $C_{22}H_{33}O_2N$.

Isolation of the active principle.—Atisine was prepared by following the method of Lawson and Topps (*loc. cit.*) with slight modification. The finely powdered rhizomes were completely exhausted with 90 per cent warm alcohol. The solvent was distilled off under reduced pressure and the brownish black mass left was then treated with 2 per cent H_2SO_4 . The acid solution was extracted several times with ether to remove oil and tarry matter. Chloroform was avoided as most of the alkaloidal salts are soluble in this solvent. The acid solution was alkalized with sodium hydroxide and the precipitated base was extracted with ether. The process of dissolution of the base in acid and further precipitation with alkali was continued till a colourless ethereal solution was obtained. The ethereal solution was washed with water till it was free from alkali. This was dried over anhydrous magnesium sulphate and ether was removed by distillation. The base was left as an amorphous colourless varnish which was only slightly soluble in water, but freely soluble in alcohol, ether and chloroform.

Pharmacology.—Antipyretic action. *Experimental.*—A large number of healthy adult rabbits (weight varying between 1.3 kg. and 1.6 kg.) was taken and their temperatures recorded. Temperature was artificially raised by giving protein shock with T.A.B. vaccine ($\frac{1}{2}$ c.c. 1/m.). A rise of 3 to 4 degrees was obtained in each case after about two hours. Atisine was administered in doses ranging from 10 mg. to 12 mg. per kilo both by mouth and intramuscularly. Two sets of controls were used: (a) One set was given aspirin (0.1 g.

per kg.); (b) The other set was not given any medicine. Temperature was then recorded every half hour till three hours after administration of the drug.

As a result of these experiments the conclusion was reached that atisine in doses ranging from 10 mg. to 12 mg. per kg. administered either orally or intramuscularly has no antipyretic effect.

General.—A large number of pharmacodynamic experiments was put up to see the effect of atisine on blood pressure. Urethanized as well as spinal animals, such as cats, rabbits and dogs, were used. It was found that atisine, when administered intravenously in doses up to as much as 10 mg. to 15 mg. per kg., produced only a slight transient fall in blood-pressure. This alkaloid, therefore, appears to be physiologically a relatively inactive substance.

II. *Xanthium strumarium* Linn.

The plant is a coarse annual herb which grows wild in Kashmir. It is an important plant as it is widely used in both the Ayurvedic and Tibbi systems of medicine as a diaphoretic and sedative and for other purposes. It has been stated that the 'young flowering tops and the two leaves immediately below' boiled in water are eaten as a pot herb by the inhabitants of certain parts of Assam. This statement requires explanation owing to the fact that the plant has been found to be poisonous to cattle and pigs in America and Australia.

Isolation of the active principle.—The endosperm of the seed, obtained by pressing out the fruit, was used for the isolation of active principle. Half a pound of the powdered seeds were exhausted with petroleum ether whereby a pale-yellow fixed oil (about 32 per cent) was obtained. The oil-free seeds were then percolated with 90 per cent alcohol. Calcium carbonate was next added to the percolate and alcohol removed under reduced pressure. The active principle being a water-soluble glucoside, the concentrated extract was treated with hot water and filtered; in this way water-insoluble impurities were eliminated. The glucoside occurring in amorphous form was further taken up in different solvents thereby eliminating still more impurities. Attempts to crystallize the glucoside, however, failed. One per cent solution of the active principle was prepared for working out its pharmacological action.

Pharmacological action.—A number of experiments was put up to observe the effect of the active principle on blood-pressure and respiration. Administration of as much as 30 mg. to 40 mg. per kg. did not produce any marked physiological effect.

A medium-sized rabbit survived without effect on the respiration and blood-pressure even after the administration of a total of 120 mg. of the glucoside. The active principle, therefore, appears to be physiologically a relatively inactive substance.

III. *Lippia nodiflora* Rich.

This is a perennial herb which grows all over India and belongs to the Natural Order *Verbenaceæ*. The drug is called 'Bukhan' in Punjabi, 'Vashira' in Sanskrit, and 'Bhuikhora' in Hindi. The plant is widely used for different purposes in different parts of the country, and is alleged to be diuretic and febrifuge. All parts of the plant are used medicinally.

Chemical investigation.—No work on the plant has previously been reported. The whole plant was powdered and successively extracted with petroleum ether, ether sulphuric, chloroform, ethyl acetate and alcohol, the percentages of the extracts after removal of the solvents being respectively 1.46 per cent, 1.0 per cent, 1.16 per cent, 0.33 per cent and 4.1 per cent. The petroleum-ether extract showed the presence of a small quantity of fixed oil and resins. The ether extract contained resins and large quantities of chlorophyll only. Even the chloroform extract did not show the presence of any alkaloid but only resins and chlorophyll. The ethyl-acetate extract showed the presence of a non-glucosidic bitter substance. The alcoholic extract contained a large amount of potassium nitrate which crystallized out when an aqueous solution of the alcoholic extract was concentrated. One hundred grammes of the powdered plant on steam distillation gave traces of an essential oil.

Isolation and purification of the bitter substance.—Three pounds of the powdered plant were exhausted with 90 per cent alcohol. The solvent was removed under reduced pressure.

The extract was mixed with calcium carbonate and extracted first with petroleum ether to get rid of petroleum-ether-soluble fraction. It was next extracted with ethyl acetate, the solvent distilled off and the extract treated several times with dilute alcohol whereby most of the chlorophyll and other impurities were removed. Finally, the residue was dissolved in alcohol and precipitated with ether. The bitter substance was thus obtained in a pure form but in very small quantities.

From the very small yield of the bitter substance it would appear that the drug cannot possess any marked degree of therapeutic activity. The presence of nitrate may, however, well account for the alleged diuretic action of the plant.

We have much pleasure in acknowledging the help and advice we have received from Colonel Sir Ram Nath Chopra, *Kt.*, C.I.E., I.M.S.(*r.*), Mr. N. N. Ghosh and Rai Sahib R. L. Badhwar.

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HÆMATOLOGICAL STUDIES IN *SILENUS (MACACUS) RHEBUS*.

Part II.

NATURE OF MALARIAL ANÆMIA IN MONKEYS.

BY

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[Received for publication, October 27, 1944.]

INTRODUCTION.

ALTHOUGH there is an extensive literature on anæmia in malaria, no exhaustive studies on this subject have been made using modern hæmatological methods. Napier (1939) studied 35 cases of malaria under hospital conditions by modern methods but he did not exclude other causes of anæmia such as hookworm infection. The investigations carried out by Fairley and Bromfield (1933) did not include estimations of the volume and hæmoglobin content of erythrocytes, while the later work of Livadas *et al.* (1939) and Bianchi (1940) did not include estimations by the newer methods of hæmatological technique. The exact nature of the anæmia caused by malarial infections has not yet been determined. Fairley *et al.* (1938) suggested that malaria might be a factor in the production of nutritional macrocytic anæmia. Napier and Majumder (1938), while investigating the anæmias of pregnancy among tea-garden coolie women in Assam, suspected that malaria might be an important factor in the ætiology of hyperchromic anæmia. No exhaustive studies have so far been undertaken on the anæmias associated with malaria in animals, although the work of Young (1937), in canaries infected with *P. rouxi*, deserves special mention. In this paper the results of investigations undertaken in *S. rhesus* infected with *P. knowlesi* are given. Studies have been made by modern hæmatological technique on monkeys suffering from acute, sub-acute and chronic infections. The results obtained have been discussed in relation to the type of anæmia occurring in animals where malaria was the sole cause of anæmia.

METHODS OF INVESTIGATION.

Observations were limited to 12 *rhesus* monkeys of which one was kept as a control and the remaining 11 were infected with *P. knowlesi*. All hæmatological investigations were made according to the methods described by Napier and Das Gupta (1942). Full blood counts were done, at intervals, on individual animals including the calculation of mean corpuscular volume (MCV), mean corpuscular hæmoglobin (MCH), and mean corpuscular hæmoglobin concentration (MCHC). Estimations were made at intervals on the normal monkey over a period of six months, and on all monkeys before infection and at intervals, sometimes daily, during the period of observation in infected animals. In the absence of hæmolysis of the blood plasma, the indirect van den Bergh reaction was also estimated.

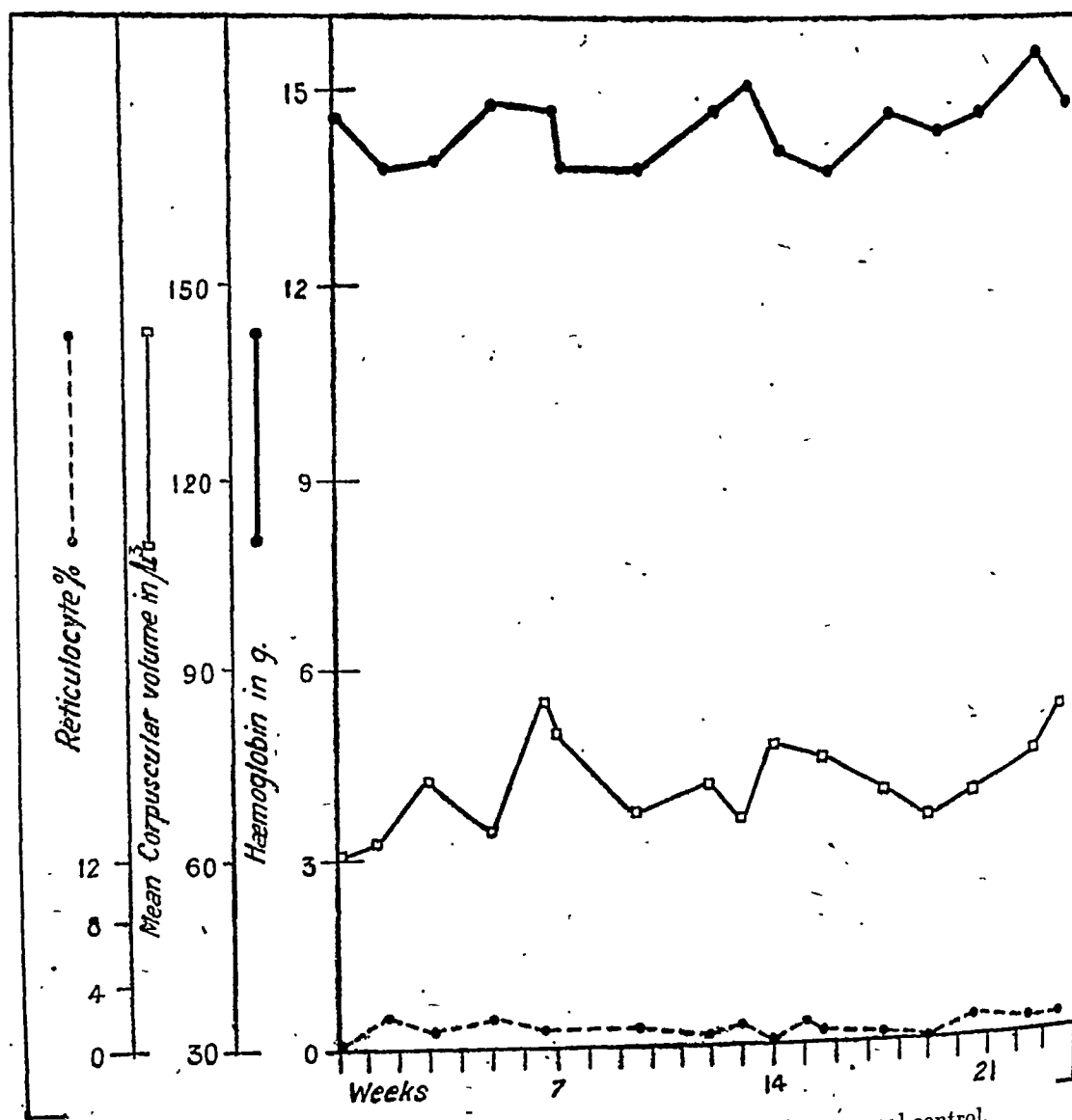
P. knowlesi in *rhesus* monkeys almost invariably causes a very acute and ultimately fatal infection, in the absence of specific treatment. An extreme degree of parasitæmia is observed in the terminal stages. By commencing specific treatment with quinine early in the course of infection, it is possible, more or less at will, to modify the acute attack into a sub-acute or chronic infection by varying the dose and frequency of administration of quinine. In the present investigation, all of the monkeys were infected by blood inoculation. One monkey was untreated and died of an acute infection four days after the parasites first appeared in the peripheral blood. Of the remaining 10, a sub-acute infection was induced by quinine

treatment in 3 and a chronic infection in 7. The 3 monkeys with sub-acute infections died in from 17 to 23 days after infection, while the 7 chronic infections were kept under observations for periods ranging from 45 to 225 days. Details of the type and intensity of infection in individual animals are given in the protocols. It was possible in this way to study the hæmatological picture in three distinct types of infection—acute, sub-acute and chronic.

RESULTS.

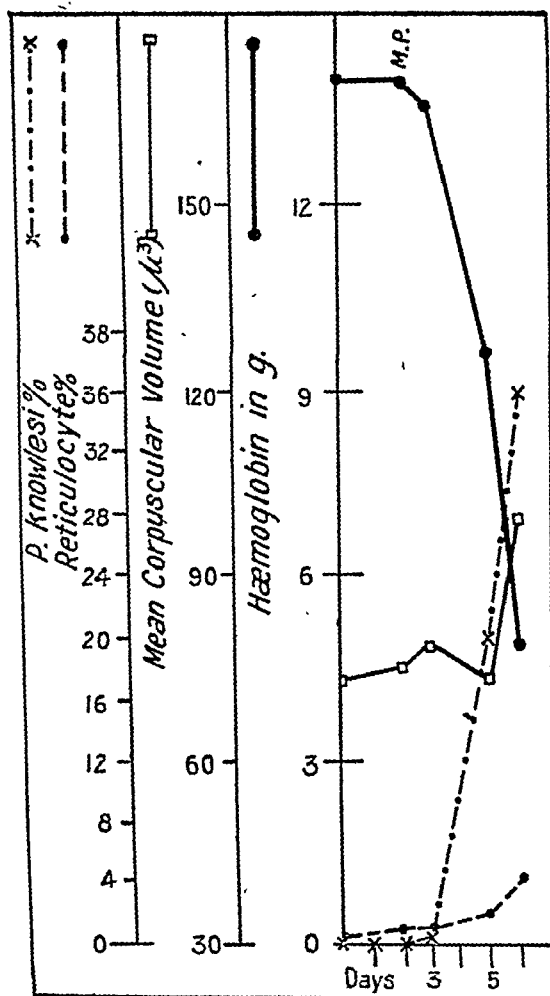
Before presenting the results, it is worth while to mention that the terms 'acute', 'sub-acute' and 'chronic', as used here, have been more or less arbitrarily chosen, having regard to the intensity of infection and the survival period of the infected animals. Further, for the sake of clearness, only three hæmatological values, namely, those of hæmoglobin, mean cell volume and reticulocytes have been charted, although full blood counts were done on every occasion.

(a) *Observations on normal S. rhesus.*—Blood counts were made on a normal specimen of *S. rhesus* (monkey No. 8) at intervals of 7 to 14 days over a period of six months. The results are recorded in Graph 1 from which it is apparent that even in a normal animal there are considerable fluctuations in normal hæmatological values:—

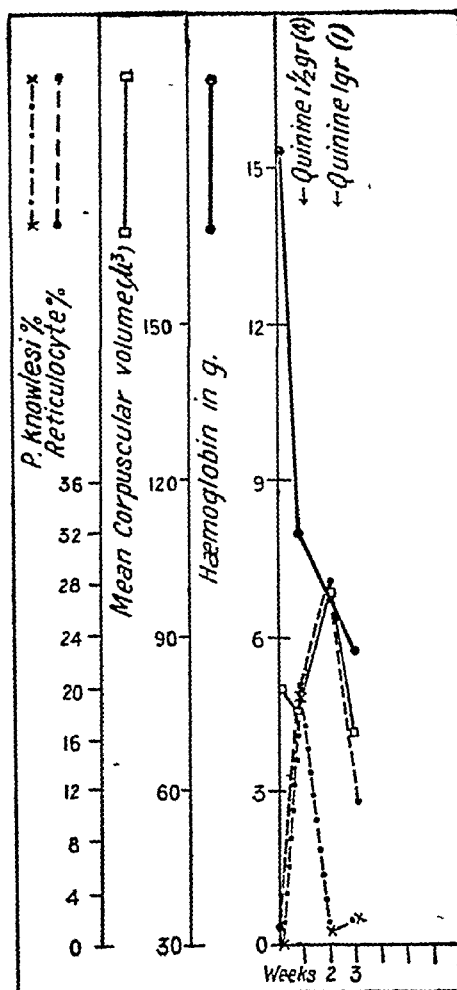


GRAPH 1.—Monkey No. 8, showing variation in blood values in a normal control.

(b) *Observations on one rhesus monkey suffering from an acute, untreated infection with P. knowlesi.*—Monkey No. 12 which is an example of acute, untreated infection with *P. knowlesi* succumbed within four days of infection. Observations were made daily and the results are recorded in Graph 2. It is clear from the graph that there was no appreciable



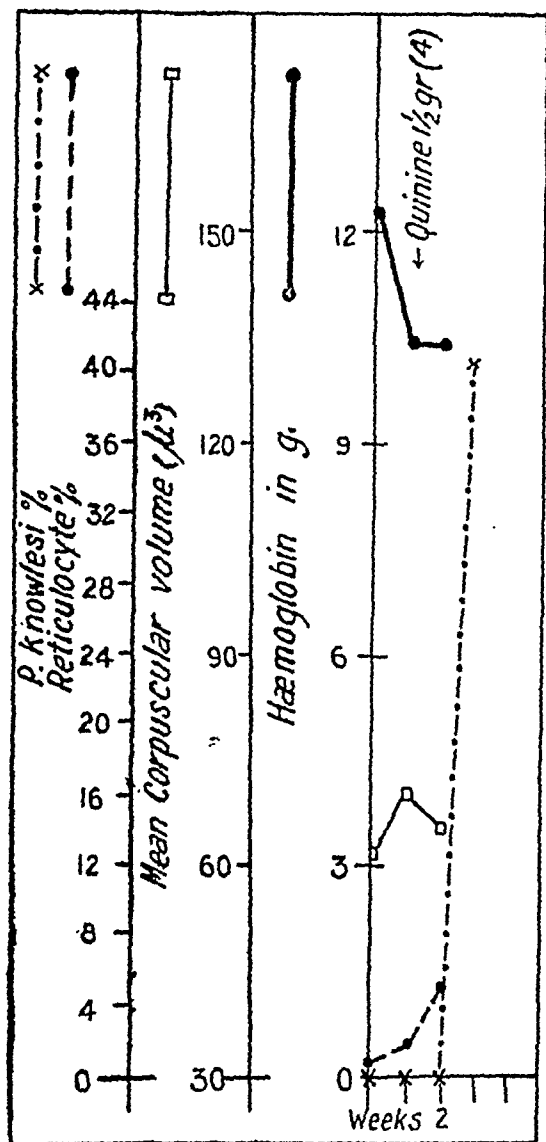
GRAPH 2.—Monkey No. 12, showing changes in blood values and rate of parasites in an acute untreated infection.



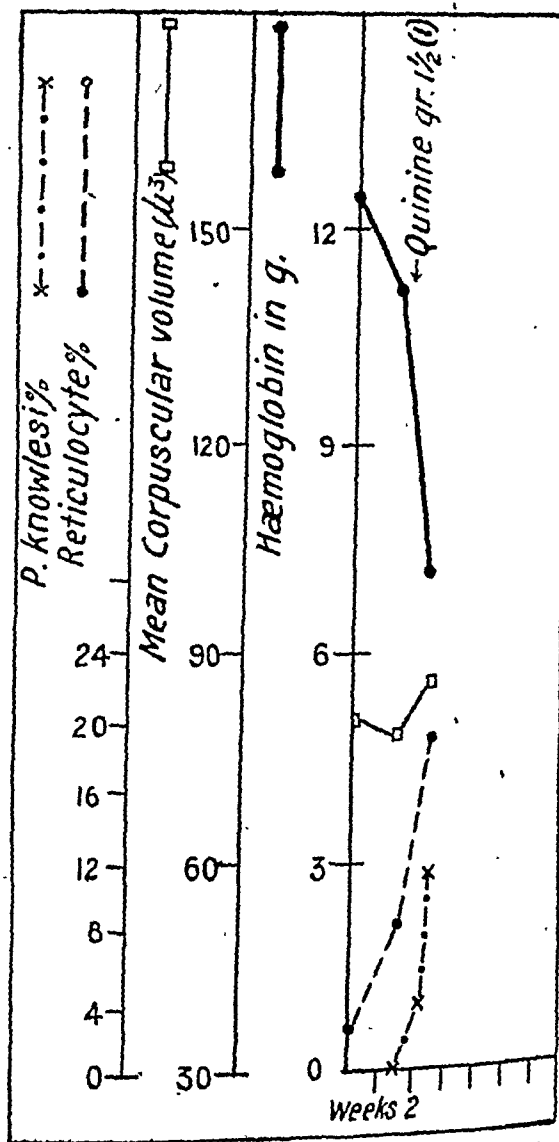
GRAPH 3.—Monkey No. 1, showing changes in blood values and the rate of parasites in a sub-acute infection. In this and the following figures arrow indicates when quinine bi-hydrochloride was given by intra-muscular injection. The dose in grains is marked against each course of injections, while the numbers in brackets refer to the number of successive days on which injections were given.

change in blood values so long as the infection rate remained less than 1 per cent of red cells. The corpuscular constants did not show much change until the last stage when a sudden upward turn occurred in the MCV curve. Reticulocytes did not show any marked increase till the last stage of infection.

(c) *Observations on 3 rhesus monkeys suffering from sub-acute infection with P. knowlesi.*—Monkeys Nos. 1, 3 and 6 inoculated with varying doses of *P. knowlesi* developed sub-acute infections. The dosage and time of administration of quinine given to these and other monkeys are explained in the legend below Graph 3. Observations on these monkeys were made at intervals of weeks and the results recorded in Graphs 3 to 5:—



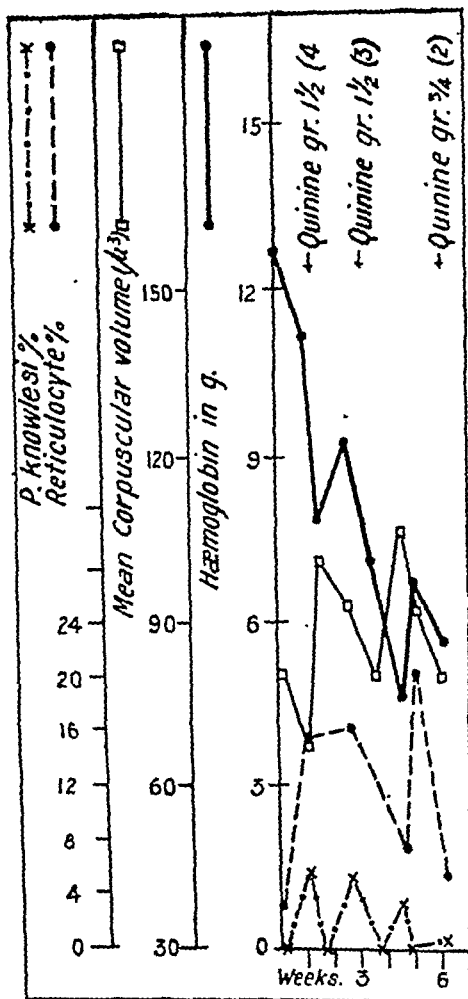
GRAPH 4.—Monkey No. 6, showing changes in blood values and the rate of parasites in a sub-acute infection.



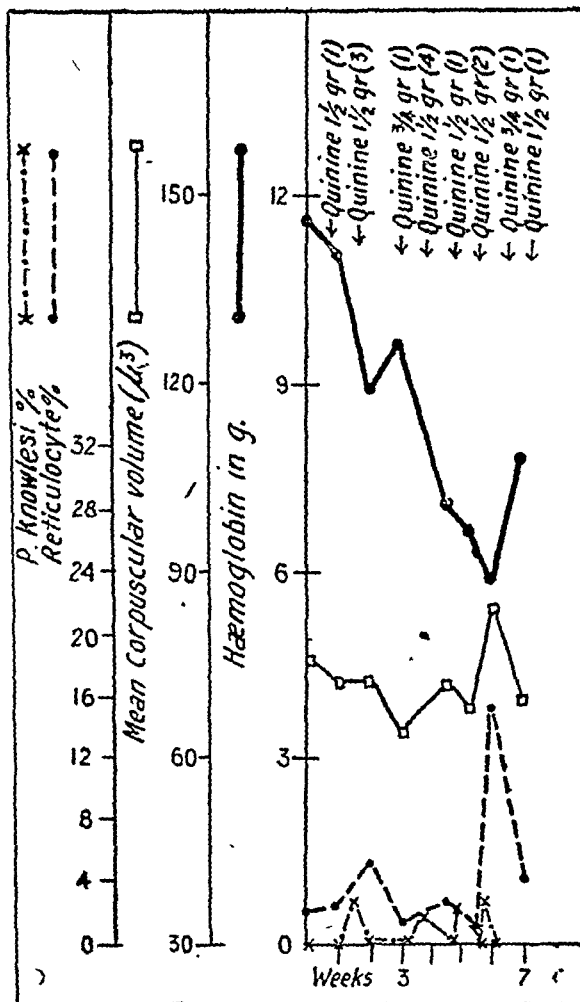
GRAPH 5.—Monkey No. 3, showing changes in blood values and rate of parasites in a sub-acute infection.

It is clear from the graphs that the course of anæmia here, though prolonged, was very much like that in acute infection in its steady downward progress. In contrast to acute infection, there was a definite tendency towards blood regeneration; as shown by the rise in the reticulocyte curve and the presence of normoblasts in the peripheral blood. In general, the corpuscular constants were not much altered in these animals.

(d) Observations on 7 rhesus monkeys suffering from chronic infection with *P. knowlesi*.—Data from monkeys Nos. 2, 4, 5, 7, 11, 22 and 23 which developed chronic infection are charted in Graphs 6 to 12. The observations on individual animals were terminated as soon as complications like diarrhoea or pyæmic abscess occurred or, in the absence of any such complications, when sufficient data had been collected.



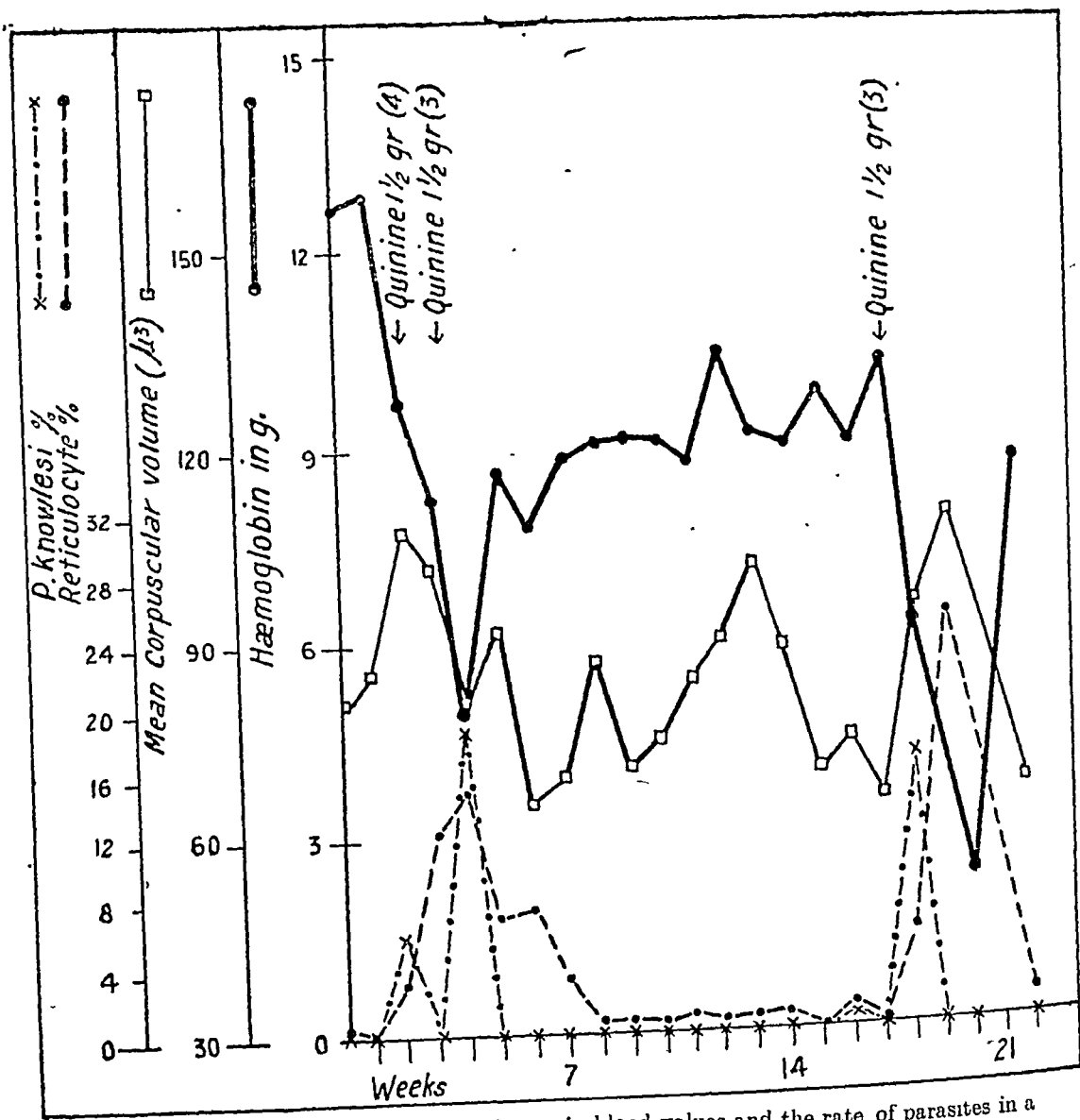
GRAPH 6.—Monkey No. 5, showing changes in blood values and rate of parasites in a chronic infection.



GRAPH 7.—Monkey No. 7, showing changes in blood values and the rate of parasites in a chronic infection.

A general examination of the graphs shows that there was a more or less sharp fall in hæmoglobin value in the first two or three weeks in all these chronically-infected animals. Their subsequent reactions varied considerably. In monkeys Nos. 5 and 7 (Graphs 6 and 7) there were comparatively small rises and falls in hæmoglobin levels at intervals of one to three

weeks; in monkeys Nos. 2 and 11 (Graphs 8 and 9) sudden drops in these levels occurred at intervals of 10 to 16 weeks, while in monkeys Nos. 4, 22 and 23 (Graphs 10, 11 and 12) there was a tendency for the hæmoglobin curve either to go up or remain more or less steady.



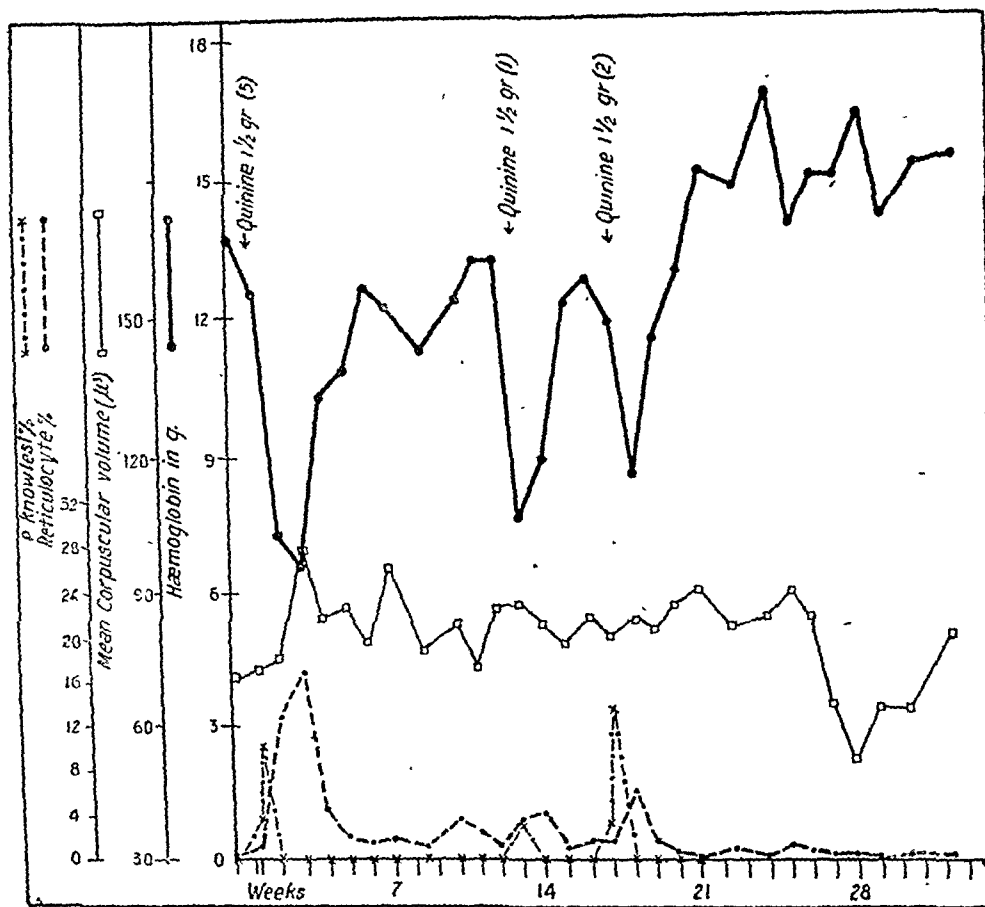
GRAPH 8.—Monkey No. 2, showing change in blood values and the rate of parasites in a chronic infection.

As shown by the reticulocyte curves and also by the presence of normoblasts, there was a marked tendency towards blood regeneration here as in sub-acute infection. Generally, the fluctuations in the reticulocyte curve and hæmoglobin curve were in opposite directions, but in no case was this relation very strict. In monkey No. 23, which was suffering from such low-grade chronic infection as to require no quinine injections, there was a sharp and sudden reticulocytosis on one occasion during the phase of steady fall in hæmoglobin level over nine weeks.

Here again, the corpuscular constants, as typified by the MCV curve, reveal no special characteristics except that the fluctuations in some of them were wider than those in acute or sub-acute infections.

DISCUSSION.

In a previous paper (Majumder and Das Gupta, 1944), it has been shown that the mean hæmoglobin and red cells of normal monkeys are 13.59 ± 1.04 g. and 5.76 ± 0.63 millions, respectively. From these figures, it is clear that a range of 16.19 g. to 10.99 g. for hæmoglobin

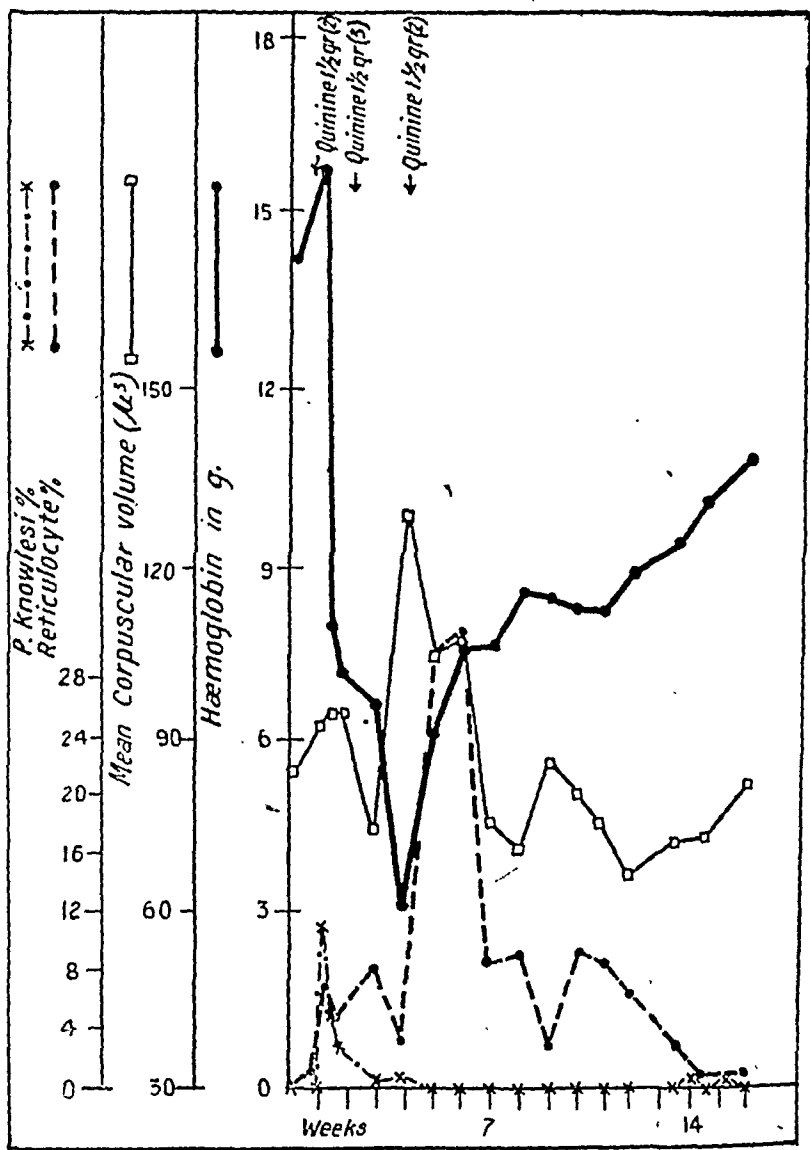


GRAPH 9.—Monkey No. 11, showing changes in blood values and rate of parasites in a chronic infection.

value and of 7.34 millions to 4.19 millions for red cells will include nearly 99 per cent of observations on normal monkeys. Accepting, therefore, 11.0 g. and 4.2 millions, as the lowest limits of normal values, we find that out of a total of 122 blood counts made on monkeys experimentally infected with malaria, 88 showed evidence of anæmia and 34 did not.

The 'non-anæmic' counts occurred either in the pre-patent or in the patent stage when only a low degree of parasitæmia was present. Anæmia, however, invariably results as soon

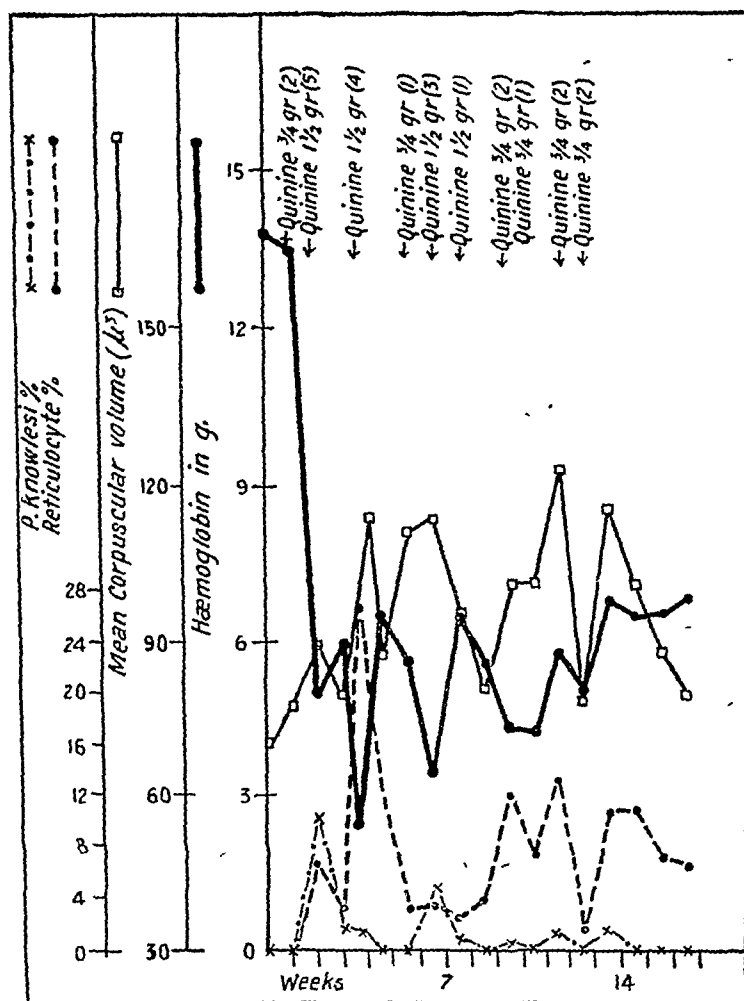
as the parasites reach a certain concentration, varying in different monkeys, but generally round about 1 per cent of red cells. It is clear from the graphs that the course of anæmia depends largely on the course of malarial infection and that even among animals exhibiting the same type of infection; reactions vary greatly from monkey to monkey. With adequate and timely quinine administration, the peripheral blood always becomes negative and with it, the condition of blood, often but not always, improves to a marked degree.



GRAPH 10.—Monkey No. 4, showing changes in blood values and the rate of parasites in a chronic infection.

The type of malarial anæmia in monkeys is best studied by comparing the mean MCV and MCH values of each animal after the onset of anæmia, with similar values on the same monkey, before it was inoculated with malarial parasites. To the differences of these means the 't' test devised by Fisher (1938) is applied. It is found that so far as the mean MCV values are concerned there is no significant change in any animal, while so far as the mean MCH values are concerned, with the exception of one monkey there is no significant change.

When, however, these corpuscular constants are projected as a dot diagram against the background of normal variations for these values, a wide scattering is noticed in the distribution. Thus, for instance, out of 88 anæmic counts, as many as 24 (about 27 per cent) have a mean corpuscular volume of $94.5 \mu^3$ or more as against only six (about 2 per cent) out of a total of 245 normal counts (*loc. cit.*). But as the increased red cell size in these 24 counts



GRAPH 11.—Monkey No. 22, showing changes in blood values and the rate of parasites in a chronic infection.

is not accompanied by any commensurate increase in hæmoglobin content of the order of $28.8 \gamma\gamma$ or more, this macrocytic tendency requires some explanation. It is to be noted that these macrocytic counts do not form the bulk of anæmic counts in any single monkey and are more or less directly related to the degree of anæmia as well as to the number of reticulocytes in blood. Dameshek and Schwartz (1938) have shown that increased reticulocytosis might lead to what they term 'pseudo-macrocytosis'. On the other hand, Elias and Kaunitz (quoted by Mohr, 1938) and later Brown *et al.* (1942) have pointed out that anoxæmia and acidosis

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SPECTRA OF HÆMOCHROMOGEN AND CYANHÆMOCHROMOGEN.

BY

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THE ABSORPTION BANDS OF HÆMOCHROMOGEN.

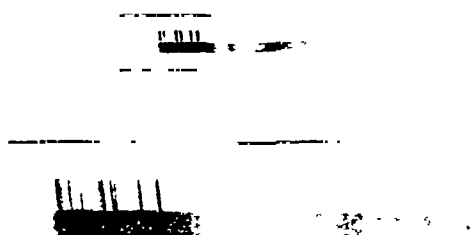
THERE is a discrepancy in the illustrations or descriptions of the spectrum of hæmochromogen given in well-known books. According to some workers the right-hand band (on the observer's right), in the green, is narrower than the left-hand band (on the observer's left), in the yellow (Sutherland, 1907; Hutchison and Hunter, 1935; Lloyd, 1935); while according to others the right-hand band is broader than the left-hand band (Plimmer, 1933; Lucas, 1935; Smith, 1938).

THE ABSORPTION BANDS OF CYANHÆMOCHROMOGEN.

The writers are not very familiar with the bands of hæmochromogen. They employ routinely the bands of cyanhæmochromogen in establishing spectroscopically the presence of blood in stains of which they test seventeen to eighteen thousand a year. Evidence of the same discrepancy also exists with respect to these bands. The right-hand band is stated and/or shown to be narrower (Lloyd, *loc. cit.*: Modi, 1943) than the left-hand band, but is known to be broader to the present writers, as shown in the following photograph taken with a spectrograph.

The process employed in taking the photograph was this:—

Forty-five mg. of dry blood were dissolved in 8 c.c. normal saline and 1 c.c. of 10 per cent KCN solution added. After about half an hour, when most of the blood had dissolved, about 0.75 c.c. of ammonium sulphide was added. After shaking well the clear fluid was transferred to a cell. The spectrum was taken within 5 minutes.



The smaller photograph is a contact print.
The larger photograph is an enlargement.

The white upright lines on the left-hand mark the red end.

The right-hand band (towards the violet end) is fainter but broader.

For studying the absorption spectrum of cyanhæmochromogen prepared in this way, a Carl Zeiss medium quartz spectrograph was used with a dispersion of about 58 Å per mm. at 5,000 Å. The source of light was a 100 watt Osram bulb which gives a continuous spectrum

of fairly uniform intensity in the required region. It was found that the absorption band on the longer wave-length side (red end) was intense and that on the shorter wave-length side (violet end) faint but broader.

The solution was examined 4 hours later with a direct-vision spectroscope. The bands were at first very faint, but again became prominent when a few drops of ammonium sulphide were added. Another photograph was then taken. In this photograph also the absorption band on the shorter wave-length side was found to be the broader of the two as before.

In routine microspectroscopy as practised in the writers' laboratory a small fragment of the stain is placed on a slide and to it are added 1 drop of a 10 per cent solution of potassium cyanide and 1 drop of ammonium sulphide and a mica cover slip applied. After locating a cherry-red spot with the microscope the eye piece of the latter is replaced by the direct-vision pocket spectroscope. The right-hand band is always broader though a part of it is rather faint. In this technique the quantity of the added reagents is greater.

The reason why the reverse is stated to be the case appears to be that in the direct-vision method the right-hand band begins to fade soon after it has become visible.

The right-hand band does not appear to be very important. 'The presence of the intense band (left hand, S. D. S. G.) is considered sufficient proof of the presence of blood, assuming that the control tests with unstained material in cases of dyed fabrics are satisfactory' (Lucas, *loc. cit.*). There is, however, no difficulty in seeing the two bands, when the specimens for the direct-vision method are so prepared that each specimen will be seen within a few minutes of its preparation, although, fallaciously, the right-hand band is likely to be believed to be narrower of the two.

SEMBLANCES OF ABSORPTION BANDS OF HÆMOGLOBIN DERIVATIVES PRODUCED BY DYES OF ANIMAL AND VEGETABLE ORIGIN.

Another discrepancy concerns the semblances of the spectrum of cyanhæmochromogen shown by cochineal, alkanet root and madder. These dyes are known to have spectra which superficially resemble those of hæmoglobulin derivatives (Lloyd, *loc. cit.*; Lucas, *loc. cit.*). They are, however, stated not to have any such spectrum when treated as a blood stain is treated for obtaining the spectrum of cyanhæmochromogen (Lucas, *loc. cit.*). The writers find that cochineal when so treated produces a spectrum which can be mistaken for that of cyanhæmochromogen. As the dyes do not give the benzidine reaction in the preliminary chemical tests they are not likely to lead to an error, because of their spectra, in the systematic examination of blood stains. The need for a critical eye in spectroscopy, however, is obvious, especially when dealing with dyed fabrics. The cherry-red colour of cyanhæmochromogen under the microscope is an important confirmatory requirement.

ACKNOWLEDGMENTS.

The writers are indebted to the Department of Biochemistry and Nutrition, All-India Institute of Hygiene & Public Health, Calcutta, for the aid given by Mr. G. Karmakar, M.Sc., in taking the photographs. They are also grateful to Professor S. Ghosh, M.Sc., of the School of Tropical Medicine, Calcutta, for the supply of cochineal and madder.

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FILARIAL INFECTION IN DHAMDA (DRUG DISTRICT, C. P.) DUE TO *WUCHERERIA MALAYI*.

BY

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(From the Filariasis Inquiry, Indian Research Fund Association, School of Tropical
Medicine, Calcutta.)

[Received for publication, December 11, 1944.]

ENDEMIC filarial infection due to *Wuchereria malayi* was recorded by the author in Patnagarh in Orissa Feudatory States (Sundar Rao, 1936) and in Ratanpur in Bilaspur district, C. P. (Sundar Rao, 1940). It was found that both these rural areas were at one time important trade centres of the ancient Hindu kings, still having their relics of a large number of tanks in each area with suitable conditions for the breeding of the mansonioides spp. of mosquitoes, viz. tanks covered with *Pistia stratiotes* and contaminated with rich organic matter. In a search for similar areas in Chattisgarh division a clue was obtained from the residents of Ratanpur that in Dhamda conditions similar to those in Ratanpur existed, Dhamda having been a trade centre closely connected with the kingdom of Ratanpur. A survey of this village was undertaken in February 1942 and the results of the survey are recorded here (cf. *District Gazetteers*, C. P., 1910).

Dhamda village in Drug tehsil is situated 21 miles to the north of Drug town which is the headquarters of the district of the same name in Central Provinces. Drug tehsil lies between 20° 51' and 21° 33' N. and 81° 6' and 81° 37' E. The area of Dhamda proper is 3,600 acres and consists of an open plain of fertile black soil alternating with sandy soil and gravel, mainly devoted to the cultivation of wheat and rice. The climate of Dhamda like that of Drug is exceptionally hot in summer and fairly cold in winter. The average annual rainfall is about 47 inches.

The village is divided into nine *mohallas* or wards, with 879 houses, mostly thatched mud huts, and has a total population of 3,628. The population consists mostly of Hindus (3,319 comprising of 37 different sects). 287 Mohammedans and 22 Indian Christians. Agriculture is their chief occupation.

There are no roads in the village except the main road from Drug which passes through Nankatti, Dhamda, Deorbija to Bemetara. There are no drains nor conservancy except hand-removal of night-soil from a few latrines attached to the district board buildings. The rest of the population use scrub jungle or open ground for defæcation. Water-supply is obtained from wells and tanks.

To determine the incidence of filarial infection in this village, population free from filarial disease was examined at night between the hours of 9 p.m. and midnight, and thick smears of peripheral blood from the finger were taken for subsequent examination. In a total of 120 persons thus examined 16 were found to show *Microfilaria malayi* infection (13.3 per cent). There was no bancroftian infection in any of them.

A house-to-house survey for filarial diseases showed that in the whole population there were 80 cases of elephantiasis of the legs or hands (2.2 per cent). The cases of elephantiasis gave a history of lymphangitis which recurred in a mild or severe form at varying intervals. Abscesses along the main lymphatic vessels were common and scars of abscesses were present in a great majority of these patients. No case of hydrocele, chyluria, lymph-varix or elephantiasis of the genitals was observed in this area.

The following species of mosquitoes were found to be prevalent in Dhamda during the period of the survey. They were kindly identified by Dr. M. O. T. Iyengar, D.Sc., Officer-in-Charge, Bengal Malaria Research Laboratory, Public Health Department, Calcutta.—

Mansonioides annulifera.
Culex fatigans.

Mansonioides uniformis.
Anopheles culicifacies.

of fairly uniform intensity in the required region. It was found that the absorption band on the longer wave-length side (red end) was intense and that on the shorter wave-length side (violet end) faint but broader.

The solution was examined 4 hours later with a direct-vision spectroscope. The bands were at first very faint, but again became prominent when a few drops of ammonium sulphide were added. Another photograph was then taken. In this photograph also the absorption band on the shorter wave-length side was found to be the broader of the two as before.

In routine microspectroscopy as practised in the writers' laboratory a small fragment of the stain is placed on a slide and to it are added 1 drop of a 10 per cent solution of potassium cyanide and 1 drop of ammonium sulphide and a mica cover slip applied. After locating a cherry-red spot with the microscope the eye piece of the latter is replaced by the direct-vision pocket spectroscope. The right-hand band is always broader though a part of it is rather faint. In this technique the quantity of the added reagents is greater.

The reason why the reverse is stated to be the case appears to be that in the direct-vision method the right-hand band begins to fade soon after it has become visible.

The right-hand band does not appear to be very important. 'The presence of the intense band (left hand, S. D. S. G.) is considered sufficient proof of the presence of blood, assuming that the control tests with unstained material in cases of dyed fabrics are satisfactory' (Lucas, *loc. cit.*). There is, however, no difficulty in seeing the two bands, when the specimens for the direct-vision method are so prepared that each specimen will be seen within a few minutes of its preparation, although, fallaciously, the right-hand band is likely to be believed to be narrower of the two.

SEMBLANCES OF ABSORPTION BANDS OF HÆMOGLOBIN DERIVATIVES PRODUCED BY DYES OF ANIMAL AND VEGETABLE ORIGIN.

Another discrepancy concerns the semblances of the spectrum of cyanhæmochromogen shown by cochineal, alkanet root and madder. These dyes are known to have spectra which superficially resemble those of hæmoglobulin derivatives (Lloyd, *loc. cit.*; Lucas, *loc. cit.*). They are, however, stated not to have any such spectrum when treated as a blood stain is treated for obtaining the spectrum of cyanhæmochromogen (Lucas, *loc. cit.*). The writers find that cochineal when so treated produces a spectrum which can be mistaken for that of cyanhæmochromogen. As the dyes do not give the benzidine reaction in the preliminary chemical tests they are not likely to lead to an error, because of their spectra, in the systematic examination of blood stains. The need for a critical eye in spectroscopy, however, is obvious, especially when dealing with dyed fabrics. The cherry-red colour of cyanhæmochromogen under the microscope is an important confirmatory requirement.

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THE INCIDENCE, TYPE AND BACTERIOLOGY OF SALMONELLA INFECTION IN THE ARMY IN INDIA.

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INTRODUCTION.

THE strides which have been made in recent years in our knowledge of the antigenic structure of the Salmonella group of organisms have resulted not only in a great increase in the number of serologically distinct types which has been recognized, and which now exceeds 120, but also to a considerable clarification of our views on the epidemiology of Salmonella disease and the type of disease resulting from infection. The bulk of this work has been carried out by Salmonella Centres, principally in Denmark and North America, which have been set up to co-ordinate the mass of dispersed and sporadic findings hitherto reported and to supply the skilled and specialized staff necessary for the accurate identification of the rarer members of the group. The principal outcome of the extension of our knowledge of the group is reflected in the change which has come about in our perception of the problems of epidemiology and of the various ways in which infection may manifest itself in man. At one time it was considered that Salmonellas could be divided fairly rigidly into two groups: those that were pathogenic for man and those causing disease in animals. Furthermore, human infection was generally regarded as displaying itself either as an 'enteric-like' fever due to *Bact. typhosum* or the 'paratyphoid' bacilli or as a simple gastro-enteritis caused by *Bact. enteritidis* or *Bact. typhimurium* or the preformed toxins of these organisms in food. These conceptions have been shown to be fundamentally unsound. Although *Bact. typhosum* and *Bact. paratyphosum A* and *B* have become very largely host-adapted to man, this adaptation cannot by any means be regarded as exclusive. Both *Bact. paratyphosum A* and *B* have on many occasions been isolated from mammals and birds and two epidemics of infection with *Para B* have been reported by Savage (1942) which probably originated from infected dogs. Spontaneous fatal infections due to *Bact. paratyphosum A* in white mice have been reported by Zozaya and Varela (quoted by Bornstein, 1943) from Mexico where human infection with this type is common. Even *Bact. typhosum*, usually regarded as an exclusively human pathogen, has been isolated from a fowl. A few other Salmonella are host-adapted to a high degree, the most typical examples being *Bact. pullorum* and *Bact. gallinarum* which cause bacillary white diarrhoea in fowl but which have also been isolated from disease in man. With regard to the remainder, some of which are commonly isolated from man—for example, *Bact. typhimurium*, *Bact. enteritidis*, *Bact. enteritidis* var. *dublin* and *Bact. cholerae-suis*—the following points should be noted:—

1. Almost without exception, Salmonella which have been proved to be pathogenic for man have also been incriminated as the cause of disease in animals. Similarly, the great majority of types primarily isolated from an animal source has now been isolated from human infections of one sort or another.
2. Figures compiled on a large scale by three Salmonella Centres showing the frequency of Salmonella types among man and animals (including fowl) in North America exhibit a marked correlation between the incidence in animals and man of any one type of more than twenty studied (Bornstein, *loc. cit.*).

Mansonioides species were found to breed in most of the big tanks. All these tanks were full of *Pistia stratiotes*. *Culex fatigans* was found to breed in small collections of water near the wells.

Mosquitoes collected from habitations from different parts of Dhamda were dissected and examined for filarial infection. Of these only *Mansonioides annulifera* showed the infection. The most common species of mosquito in the area was *M. annulifera*. It is apparently the most important transmitter of filarial infection in Dhamda.

SUMMARY.

Dhamda in Drug district (C. P.), a moderately big village, has endemic filariasis, the infection being due to *W. malayi*. The microfilaria rate is 13·3 per cent and the filarial disease rate is 2·2 per cent. Elephantiasis of the genitals, hydrocele and chyluria are entirely absent. The mosquito survey showed a prevalence of *Mansonioides* species. *Mansonioides annulifera* is the commonest mosquito in Dhamda and appears to be the chief transmitter of filarial infection. The breeding places are the large tanks covered with *pistia*.

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were received for verification of diagnosis. Of these, 1,299 were identified as *Bact. typhosum*. This leaves a balance of 548 organisms of other types. Thus, thirty per cent of all organisms of the Salmonella group received by us have been other than *Bact. typhosum*. These figures are the average obtained over a period of four years during the greater part of which the size and living conditions of the Army in India have, on the whole, undergone substantial change. They do not, as they stand, take into account relative variation in the incidence of infection by the various types during this period. This variation is shown and represented graphically in Table I and the Graph respectively :—

TABLE I.

Year.	STRAINS OF <i>Bact. typhosum</i> .		STRAINS OF OTHER SALMONELLAS.		Total number.	Other Salmonellas. Percentage of total.
	Number.	Percentage increase on previous year.	Number.	Percentage increase on previous year.		
1941	166	17.8	72	9.1	238	30.4
1942	318	92.7	66	—8.3	384	17.2
1943	394	23.9	170	157.5	564	30.1
1944	421	6.6	240	40.6	661	36.3

TOTAL 1,847 AVERAGE 29.7

The number of isolations of *Bact. typhosum* rose steeply from 1941 to 1942 but thereafter the percentage increase has progressively declined. This increase is purely in numbers of cases and is due to the rapid expansion of the Army in India following the Japanese threat at the beginning of 1942. The incidence of enteric per 1,000 of the Army in India has actually fallen throughout this period.

Isolations of Salmonella other than *Bact. typhosum* did not show any corresponding rise from 1941 to 1942; in fact, there was a slight but probably insignificant fall in their number. From the end of 1942 onwards, however, their number started to rise and has continued to do so progressively up to the time of writing, despite the tailing off in the increase in *Bact. typhosum* isolations. The probable reason for this continued increase will be dealt with later. It must be pointed out that the original figures for 1941 were complicated by the large numbers of Italian prisoners of war entering India in that year. Enteric was prevalent among these men. A considerable number of isolations of *Bact. paratyphosum B* were received from them but this organism, judging by subsequent findings, failed to establish itself outside the camps where the Italians were interned and was later replaced by a *Bact. paratyphosum A* infection. In view of the abnormal conditions of active immunization and environment under which these men existed and of the fact that they must have imported much of their source of infection with them, as is shown by the high incidence of *Bact. paratyphosum B* infection among them, all isolations received from them

Where the infectivity rate in animals is high the incidence in man is correspondingly great. Where the incidence in animals is moderate or low, the incidence in man follows suit.

3. There is little doubt that non-host-adapted organisms may become temporarily host-adapted during the course of an epidemic and that this adaptation may be associated with a change in the character of the disease. This has been observed in the case of *Bact. paratyphosum B* which may be regarded as intermediate in adaptability between *Bact. typhosum* and the majority of other Salmonellas. In some outbreaks of infection with this organism the early cases have suffered only from gastro-enteritis, while typhoid-like cases developed later in the epidemic (Savage, *loc. cit.*). In occasional epidemics only gastro-enteritis cases have been reported (Feemster and Anderson, 1939). It is fair to assume that, apart from *Bact. typhosum* and the majority of infections with *Bact. paratyphosum A* and *B*, animals form the reservoir of Salmonellas and that human infection is derived, in the main, from the contamination of foodstuffs with animal excreta. The human carrier should not be forgotten as an important secondary method of spread. These types are not host-adapted to any extent but, by successive passage from man to man, their pathogenic rôle may alter and give rise to infection of a more invasive nature.

The object of the next section of this paper is to present a survey of Salmonella infection among Army personnel in India. So far as we are aware such a survey has not hitherto been published.

INCIDENCE AND TYPE.

One of the duties of the Serological Department of the Central Military Pathological Laboratory (India) (formerly the Enteric Laboratory, Kasauli) is the confirmation of bacteriological diagnoses of all organisms of the Salmonella group isolated by military laboratories in India and South-East Asia Commands. Our figures should, therefore, be representative of the state of affairs obtaining in the Army throughout the whole of India and Ceylon but the number of organisms received is, in most cases, insufficiently great to warrant the stressing of any apparent regional prevalence as being significant. Criticism of the statistical significance of our figures may be forthcoming on the following grounds:—

1. Not only do all laboratories not send for verification the Salmonellas isolated by them but it is probable that those laboratories that do utilize our services fail to send all their primary isolations. Steps have already been taken to remedy this.
2. The organisms received for confirmation of diagnosis are not a fair sample of those isolated since there is a tendency to forward only those types most difficult of identification. This will tend to give bias to the rarer types and to diminish the apparent incidence of the commoner types.
3. The number of cases from whom the causative organism is isolated do not necessarily form an accurate index of the true number of infections. They do, however, coincide with the number of bacteriologically proven cases which, taking into account the considerable possibilities of error when diagnosis is made on clinical and serological grounds alone, form the only true basis from which valid conclusions may be drawn.

Criticisms 1 and 2 are valid, but are equally applicable to all extensive surveys which by their nature usually preclude close co-operation with the clinician. Our figures have the merit of dealing only with primary isolations and are not confused by the error of duplication.

The data here published cover the years 1941, 1942, 1943, 1944 and the first six weeks of 1945. During this period a total of 1,847 organisms of the Salmonella group

An explanation for the continued rise in extra-typhoid isolations can be found in an analysis of the types of these strains. Such an analysis of 548 strains, exclusive of *Bact. typhosum*, is given in Table II:—

TABLE II.

Organism (excluding <i>Bact. typhosum</i>).	NUMBER RECEIVED FOR VERIFICATION DURING				Total.
	1941.	1942.	1943.	1944 and 6/52 1945.	
<i>Bact. paratyphosum A</i> ...	60	52	93	76	281
<i>Bact. paratyphosum B</i> ...	5	0	2	0	7
<i>Bact. paratyphosum C</i> ...	4	4	20	82	110
<i>Bact. enteritidis</i> ...	0	7	48	56	111
<i>Bact. enteritidis</i> var. <i>dublin</i> ...	0	0	2	12	14
<i>Bact. typhimurium</i> ...	3	3	2	10	18
Miscellaneous ...	0	0	3	4	7
TOTAL ...	72	66	170	240	548

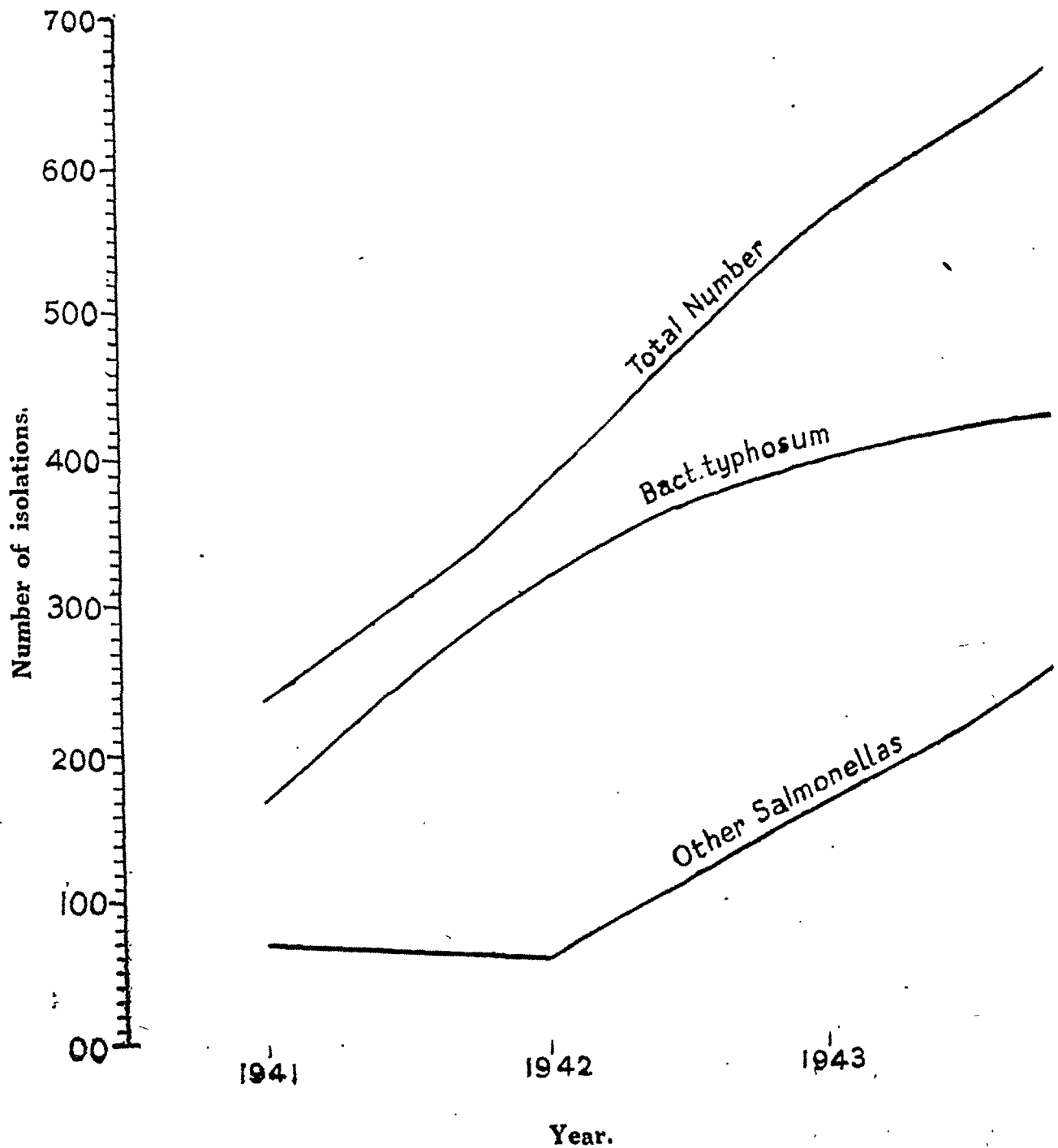
The most interesting features of Table II are the figures relating to *Bact. paratyphosum C* and *Bact. enteritidis*. Whereas the apparent incidence both of *Bact. typhosum* (see Table I) and of *Bact. paratyphosum A* infection does not show any marked aggregate variation which cannot be accounted for by the increase in size of the Army in India over the four-year period, that of *Bact. paratyphosum C* and *Bact. enteritidis* shows a significant and progressive rise. It is here that we find the explanation of the continued rise of extra-typhoid infection and the divergence of its rate of increase from that of *Bact. typhosum*. Another point of interest is the infrequent occurrence of *Bact. paratyphosum B* which, in India, may be regarded as a rare *Salmonella*. The number of strains of *Bact. enteritidis* var. *dublin* and of *Bact. typhimurium* are too small to permit any conclusions as regards incidence to be drawn.

Isolations under the heading 'Miscellaneous' comprise two strains of *Bact. anatum* from blood culture in man, two strains of monophasic *Bact. cholerae-suis* (var. *kunzendorf*) derived from blood culture, one strain of diphasic *Bact. cholerae-suis* isolated from pus, one strain of *Bact. virchow* in pure culture from an infected hæmothorax and one strain of *Bact. litchfield* from a sick duck. After the analysis of our figures had been made, a strain of *Bact. moribificans bovis* was received for identification which had been isolated from an unspecified source in a case of human infection.

The anatomical sources from which *Salmonella* received during the years 1943 and 1944—the only years for which these figures are available—were isolated are shown in Table III:—

have been excluded from our figures. Allowing for the facts quoted above, it seems reasonable to infer that not only are infections due to Salmonellas other than *Bact. typhosum* more prevalent than is suspected but that their relative incidence is increasing. Such a

GRAPH.



deduction would be valid if it is allowed that, during the period under survey, neither the quality nor the assiduity of the pathological services feeding the Central Laboratory has undergone substantial change—an assumption which we believe to be warranted.

Up to May 1943, when the policy concerning the disposal of British military enteric patients was changed, part of the duties of the Enteric Laboratory, Kasauli, was the clinical and bacteriological control of all British military personnel in India who were considered as convalescent from an attack of fever of enteric type. Of 237 of these patients dealt with during 1942 and the early part of 1943 it was found that only some 33 per cent had been diagnosed by the isolation of the causative organism, the remainder having been labelled 'unspecified enteric' on symptomatic grounds, with or without the assistance of a suggestive Widal reaction. It is probable that a proportion of these latter cases had never suffered from enteric fever at all. At the time that this state of affairs became apparent, one or both of two factors were considered primarily responsible for the unexpectedly low percentage of cases in which the diagnosis was unequivocal. The first of these was failure on the part of the physician to perform early blood culture in all cases of undiagnosed pyrexia, either because a possible diagnosis of enteric was not considered until investigation into the more common causes of fever in the tropics had proved abortive or because a high proportion of cases had not become hospitalized under conditions where adequate laboratory facilities existed during the first week or more of their attack.

• During the first week after onset of enteric fever the frequency with which *Bact. typhosum* may be isolated from the blood is about 90 per cent, falling to rather more than 70 per cent early in the second week, to 60 per cent in the middle of the third week and to 40 per cent in the middle of the fourth week (Topley and Wilson, 1936). The second factor considered of importance was the entire absence in military laboratories in India of a good selective medium for the isolation of organisms of the *Salmonella* group from the stools of those patients in whom diagnosis by blood culture had been missed. This is now being rectified. If we accept the former premise that only one-third of cases of presumptive enteric fever in the Army in India is diagnosed bacteriologically, the figures in Table III relating to *Bact. typhosum* and *Bact. paratyphosum A* amply support our second assumption that the failure of bacteriological diagnosis in the remaining cases lies in the failure to isolate the causative organism from the stools. Some ninety per cent of primary isolations of these two organisms are from the blood and more than half the remainder from the urine, internal organs or exudates from which culture presents no difficulty. Only 3.9 per cent of all primary isolations of these two organisms were from the faeces.

In connection with Table II, attention has been drawn to the high and increasing incidence of infection with *Bact. enteritidis*. In fact, figures for 1944 show that this organism and *Bact. paratyphosum C* are competing strongly with *Bact. paratyphosum A* for second place in the causation of *Salmonella* disease in the Army in India. *Bact. enteritidis* has long been regarded, together with *Bact. typhimurium*, as the classical aetiological agent in bacterial food-poisoning with gastro-enteritis in man. Descriptions of cases in whom *Bact. enteritidis* has displayed an invasive rôle are extremely rare in medical literature. Since White (1929) first demonstrated the antigenic differences between *Bact. enteritidis* (*gertner*) and *Bact. enteritidis* var. *dublin*, most of the strains incriminated as the cause of an invasive type of disease have been identified as *dublin* variants. Moreover, the majority of these cases have occurred in infants and young children who are not only very susceptible to *Salmonella* infection in general but in whom the well-known clinical differences between human infections with *Bact. paratyphosum B* and *Bact. typhimurium* are not very marked (see Bornstein, *loc. cit.*). Reference to Table III will make it clear that, in India, the vast majority (87.5 per cent) of isolations of *Bact. enteritidis* in man have been from blood culture, while the greater part of the remainder of isolations of this organism has been from situations which presume for it an invasive rôle. Less than four per cent of isolations have been from the faeces. Moreover, all these isolations have been from adult males who have been considered suitable for military service. The number of strains of *Bact. typhimurium* verified by this Laboratory has been small. This organism, too, is generally regarded as being responsible for a simple gastro-enteritis resulting from the consumption of infected food. It will be seen from Table III that, of twelve strains isolated, ten were obtained from blood culture and only two from faeces. Nine out of the ten invasive strains were isolated from cases in a small epidemic of continuous fever occurring in Karachi.

TABLE III.

•Sources of *Salmonella* strains received during 1943 and 1944.

Organism.	NUMBER OF STRAINS ISOLATED FROM							Total number of strains.	Percentage of total of strains isolated from blood.
	Blood.	Feces.	Urine.	Bile.	Pleural fluid.	Spleen.	Pus.		
* <i>Bact. typhosum</i>	27	27	11	2	...	5	811	90.0
<i>Bact. paratyphosum A</i>	146	11	7	1	1	166	88.0
<i>Bact. paratyphosum B</i>	1	1	2	...
<i>Bact. paratyphosum C</i>	87	2	3	4	5	102	85.3
<i>Bact. enteritidis</i> ...	91	4	2	3	1	2	1	105	87.5
<i>Bact. enteritidis</i> var. <i>dublin</i> ...	14	14	100.0
<i>Bact. typhimurium</i>	10	2	12	83.4

* Three strains isolated from well and swimming-pool waters and included in Table I are excluded from these figures.

In all strains this antigen was present in VW form in that (O) agglutination was impaired either not at all or only to a slight degree. Agglutination, however, is usually only to a fraction of the titre of the Vi serum. Slide agglutination using pure Vi serum is the standard technique employed in testing for Vi antigen. In a considerable proportion of strains it has been found that, using this method, no agglutination occurs when 24-hour cultures are used as agglutinin. Agglutination, however, is invariable when *logarithmic* phase agar-slope cultures are employed.

Bact. enteritidis.

One hundred and eleven strains of this organism have been received since the beginning of 1942. All, with one exception (see 'Anærogenic strains' below), were biochemically typical in that both rhamnose and arabinose were fermented. *Dublin* variants attack rhamnose only. All strains, both *gartner* and *dublin*, fermented dulcitol but it was noticed that, while *dublin* strains invariably fermented this sugar during the first 24 to 48 hours of incubation, fermentation by *gartner* strains did not occur until after the fifth day and frequently much later. With one strain acid was not produced until the seventeenth day of incubation. This is considered a point of considerable differential value and one which, so far as we know, has not been reported before.

It has been shown in connection with Table III that over 96 per cent of strains of *Bact. enteritidis* were isolated from blood or from other sources indicative of invasion in cases of continuous fever in man. In view of this marked departure of the organism from its usual pathogenic rôle it was considered worth while examining the strains at our disposal for the presence of an additional antigen analogous to the Vi antigens described by Felix and Pitt (1936) in *Bact. paratyphosum* A and B and in *Bact. typhimurium*. Felix and Pitt failed to detect the presence of any such antigen in the strains of *Bact. enteritidis* they examined but it is probable that these strains were either of animal origin or derived from gastro-enteritis in man. Rabbits were immunized with two freshly isolated strains of *gartner* and the resulting antisera absorbed with homologous suspension which had been treated with 5 per cent HCl for 48 hours followed, after neutralization, by heating for two hours at 94°C. Such treatment should have no effect on the heat and acid-stable (O) antigen. After absorption, a residual agglutinin of titre 160 to 320 was left in both sera against the immunizing suspension, alcoholized with absolute alcohol for 10 minutes to destroy flagellar agglutinability. The agglutinability of fresh, alcoholized suspensions by these sera was halved after heating at 94°C. for 15 minutes, although control experiments showed that the sensitiveness to agglutination by 'stock' *enteritidis* (O) antiserum was unaltered by heating or by treatment with HCl followed by heating. When a slide technique was used, while unheated suspensions reacted strongly with the absorbed sera, only a trace of agglutination or, more usually, none at all was observed with the same suspension after heating. The two absorbed sera were tested by the slide method against 14 strains of *Bact. enteritidis*. Thirteen of these strains, isolated from blood culture, reacted strongly while the fourteenth strain, isolated from the stool of a case of gastro-enteritis, did not react at all. Five strains of the *dublin* variant also failed to agglutinate as did *Bact. enteritidis* vars. *moscow* and *rostock*, three strains of *Bact. typhosum* and several strains each of *Bact. paratyphosum* A, B and C. Mouse protection experiments have not been performed. This work was carried out at Kasauli, a cool hill station situated at an altitude of 6,000 feet above sea-level in the Himalayan foot-hills. The Laboratory subsequently moved to Poona where the climate is considerably warmer. Here we have met with consistent failure in our efforts to confirm these experiments. Whether this is due to failure of rabbits to respond so well to weak antigenic stimuli under warm environmental conditions, to a change in the strain of rabbit used or to some other factor is not known. It has been noticed that, using the same strains for injection, (O) *Salmonella* titres obtained in Poona have, on the whole, been significantly lower than those regularly obtained at Kasauli. Moreover, while no difficulty was encountered at Kasauli in producing strong *streptococcal* grouping sera, rabbits inoculated at Poona have responded only very weakly or not at all. During

the course of this latter work several antigenic differences between strains of *Bact. enteritidis* have become apparent. Kauffmann (1941) has demonstrated that both the *Salmonella* (O) factors I and XII are subject to a type of variation reminiscent of flagellar diphasic variation. In the case of factor XII which is shared by *Bact. typhosum*, *Bact. enteritidis* (all variants) and *Bact. paratyphosum A* and *B*, three components, XII₁, XII₂, XII₃, have been demonstrated. Only component XII₂ is subject to variation. When this component is dominant it appears appreciably to 'mask' the other two components so that strong agglutination occurs with a XII₂ serum but only weak and low titre agglutination with sera against XII₁ and XII₃. The converse of this occurs when XII₂ is recessive. Kauffmann (*loc. cit.*) has shown this variation to occur in *Bact. typhosum* and in five strains of *Bact. enteritidis* but states that variation in factors I and XII occurs quite independently. Antigenic analysis of 38 of our *gärther* strains strongly suggests that two distinct antigenic (O) types exist and that both these types may differ from *dublin* strains which appear to be antigenically more homogeneous. Roughly one-third of strains has been shown to possess the (O) factor I and possession of this factor appears to be correlated with either complete absence or recessiveness of component XII₂. The remaining two-thirds of strains, which lack factor I, possess component XII₂ but appear to be deficient in one of the other two components—probably XII₃. Provisional antigenic formulæ allotted to these types are:—

Type A—I, IX, XII₁, XII₃ (? heat-labile antigen).

Type B—IX, XII₁, XII₂ (? heat-labile antigen).

This work is not yet complete and will be described more fully elsewhere at a later date. A differentiation of *Bact. enteritidis* into two clear-cut types which can readily be distinguished by slide agglutination with absorbed serum may be of some epidemiological value.

It is stressed that the components of factor XII cannot, as their nomenclature implies, be regarded as minor antigens of little importance since they are highly antigenic and are probably responsible for the considerable degree of cross-immunity that exists between *Bact. typhosum* and *Bact. paratyphosum A* and *B* (Longfellow and Luippold, 1942).

Anærogenic Salmonella strains.

Anærogenic variants of five common ærogenic types have been described in the literature to date. These are *Bact. paratyphosum A* (see Nabih, 1941), *Bact. paratyphosum B* (Warren and Iredale, 1934), *Bact. paratyphosum C* (Nabih, *loc. cit.*), *Bact. enteritidis* (Bruce and Dascomb, 1943) and *Bact. typhimurium* (Stone, 1943). Sandiford (1944) records that, although food-poisoning due to anærogenic variants of *Bact. typhimurium* is rare in Britain, such strains are, with the possible exception of *staphylococci*, the commonest organisms isolated in food-poisoning epidemics in Egypt. Anærogenic variants of *Bact. suispestifer* have also been described (Savage and White, 1925).

In our series we have encountered one strain of *Bact. paratyphosum A*, one strain of *Bact. enteritidis* and three strains of *Bact. enteritidis* var. *dublin* which failed to produce any trace of gas in the sugars which they fermented. Three additional strains of *Bact. paratyphosum A* have been isolated which, although producing no gas in peptone-water-sugars, became ærogenic when cultured in sugars prepared in nutrient broth instead of in peptone-water. It is considered that no *Salmonella* should be labelled as a true anærogen unless it has failed to produce gas when grown in nutrient broth (Lemco meat extract and peptone) to which a fermentable carbohydrate has been added. The three anærogenic *dublin* strains were derived from cases of continuous fever between whom there can have been no epidemiological connection. So far as we know, anærogenic strains of *Bact. enteritidis* var. *dublin* have not previously been described. Such anærogenic strains may give rise to considerable confusion in diagnosis unless their occurrence is borne in mind since preliminary identification of organisms of the *Salmonella* group is based on biochemical reaction and failure to produce gas together with inagglutinability by *Bact. typhosum* (H), (O) or Vi serum. may produce the impression that the organism is not a member of the group at all. In the case

of *Bact. enteritidis* a diagnosis of *Bact. typhosum* is very likely to be made in view of the similarity of the (O) antigens of the two organisms.

Naturally occurring non-motile Salmonella variants.

The isolation of true (O) variants of *Salmonellas* from disease in man is rare and the complete absence of flagella may give rise to difficulties in bacteriological diagnosis. The criteria adopted to establish the permanence of the absence of flagella have been as follows:—

1. Failure to show any evidence of motility microscopically and failure to induce motility by repeated subculture in broth, both at room temperature and at 37°C., and by attempted passage through semi-solid agar.
2. Complete inagglutinability by (H) antiserum.
3. Inability to absorb agglutinin from an (H) antiserum to the same type.
4. Failure, on injection into rabbits, to stimulate the production of (H) agglutinins against itself or against motile strains of any other organism of the (O) subgroup to which it belongs.

One strain of *Bact. paratyphosum A* and one strain of *Bact. paratyphosum C* have been encountered which fulfil these criteria. These strains have been in constant use for about nine months and have shown no evidence of flagella formation. They have proved useful in the preparation of agglutinable (O) suspensions and (O) antisera for issue to laboratories in India and overseas. The diagnosis of the *Bact. paratyphosum A* strain presented no difficulty since the (O) antigen of this organism is unique in containing factor II. Such, however, was not the case with *Bact. paratyphosum C* since 15 other organisms share with it a common (O) antigen. Type diagnosis was based on biochemical reaction and on the presence in the strain of Vi antigen. One other presumptively non-motile strain of *Bact. paratyphosum C* has recently been received but has not yet been exhaustively studied.

Uncommon Salmonella types.

Bact. anatum.—The two strains of this organism identified were isolated from blood culture in man. Four variants of this type have been described, all of which possess the same somatic and flagellar specific phase antigens but differ in a component of their flagellar group phase antigen. They are *Bact. anatum* (III, X, XXVI: e, h; 1, 6), *Bact. meunster* (III, X, XXVI: e, h; 1, 5), *Bact. nyborg* (III, X, XXVI: e, h; 1, 7) and *Bact. vejle* (III, X, XXVI: e, h; 1, 2, 3). *Bact. anatum* was originally isolated from an epizootic intestinal infection in ducks. All these variants have, however, been isolated from gastro-enteritis in man. So far as we are aware, this is the first recorded instance of *Bact. anatum* producing an invasive type of disease in man. Greifinger and Silberstein (1944) described an outbreak of 115 cases of acute gastro-enteritis resulting from bacterial food-poisoning in American Army personnel in whom *Bact. anatum* was the causative organism in 44 cases (38·2 per cent) though in all but four cases it was associated either with *Bact. typhimurium* or *Bact. oranienburg*. They record that sulphasuxidine was highly effective in clearing the stools of *Bact. anatum* and, in less degree, of *Bact. oranienburg*. No isolation from blood culture was described. Bacteriological diagnosis of our strains was established in three stages:—

1. The (O) antigen was shown to be identical with that of *Bact. london* by reciprocal absorption tests and, therefore, to contain the factors III, X, XXVI.
2. The diphasic nature of the (H) antigens of the strains was determined. Reciprocal absorption tests showed that, in the specific phase, the (II) antigen was identical with that of *Bact. newport* and, therefore, contained the factors e, h.

3. The (H) group antigen was agglutinated by antiserum against the (H) group phase of *Bact. typhimurium* (1, 2, 3) and *Bact. cholerae-suis* (1, 5). Absorbed factor-specific sera against factors 2, 3, 5 and 7 did not agglutinate the strains while anti-factor 6 did. The formula of these strains was thus determined as III, X, XXVI: c, h; 1, 6.

Bact. virchow.—One strain was recently isolated in pure culture from an infected hæmothorax. This is the first recorded isolation of this organism in India and the fourth altogether. The three previous recoveries have been from gastro-enteritis, sub-chronic enteritis and septic (mixed) infection in man (see Bornstein, *loc. cit.*). *Bact. virchow* possesses the formula VI, VII: r; 1, 2, 3. Our strain was shown, by reciprocal absorption tests, to share its (O) antigen with *Bact. paratyphosum* C (VI, VII), its (H) specific antigen with *Bact. heidelberg* (r) and its (H) group antigen with *Bact. typhimurium* (1, 2, 3). The organism did not possess the Vi antigen of *Bact. typhosum*.

Bact. litchfield.—This strain was isolated from a sick duck. It is the fifth isolation reported in the literature. The type was first described in 1940 when two strains were isolated from infected turkeys in America. A *Salmonella* recovered from a case of food-poisoning in man in 1932 and not previously identified was then found to be identical with these two strains (Edwards and Bruner, 1940). Another strain has been reported as having been isolated from a man in Florida by Galton and Quan (1943). *Bact. litchfield* has the formula VI, VIII: l, v; 1, 2, 3. Our strain was shown, by reciprocal absorption tests, to share its (O) antigen with *Bact. newport* (VI, VIII), its (H) specific antigen with *Bact. london* and *Bact. panama* (l, v) and its (H) group antigen with *Bact. typhimurium* (1, 2, 3). Vi antigen is described as having been extracted from *Bact. newport* (Rouchdi, 1938). This antigen was not detected in our strain of *Bact. litchfield*.

Bact. morbificans bovis.—This strain was obtained from an unspecified source in disease in man. It has not been included in our aggregate figures since it was identified recently during a period not covered by these figures. The type was originally isolated from a sick cow but has since been found to be causative of gastro-enteritis and continuous fever in man. It possesses the formula VI, VIII: r; 1, 5. Vi antigen was not detected in our strain.

DISCUSSION.

In spite of many and detailed reports from *Salmonella* Centres and other sources in America, Europe and elsewhere, comparative figures of the types causing infection in different parts of the world are difficult to obtain because of the exclusion of the commoner regional *Salmonellas* from many records and of the inclusion in others of strains isolated from animals as well as from man. •Over the period covered by this survey, *Bact. paratyphosum* A has, next to *Bact. typhosum*, been the most consistently prevalent cause of *Salmonella* disease in man in India. Sporadic and usually isolated cases of infection with this organism, although uncommon, are world wide in their occurrence but they do not, as a rule, attain epidemic proportions except in tropical zones. •In Western Europe and North America, if *Bact. typhosum* is excluded, *Bact. paratyphosum* B is by far the commonest cause of fever of enteric type. In contradistinction, this organism is so infrequently isolated in India that it may be regarded as a rare *Salmonella*.

Until the beginning of 1943 no *Salmonella* other than *Bact. typhosum* and *Bact. paratyphosum* A appears to have been of importance in India. Considerable interest attaches, therefore, to an apparent marked rise in incidence of *Bact. paratyphosum* C and *Bact. enteritidis* infection in India during 1943 and 1944. In the latter year the number of strains of *Bact. paratyphosum* C exceeded those of *Bact. paratyphosum* A for the same year while the figures for *Bact. enteritidis* equalled the average for *Bact. paratyphosum* A during 1941 and 1942. *Bact. paratyphosum* C is common in Eastern Europe and has been reported as prevalent in British Guiana. It is one of the few members of the *Salmonella* group which appears to be an almost exclusive human

pathogen. This would presume for it a mode of spread involving only the human case and the human carrier. Of the 75 strains received by us during 1944 only seven had been isolated from Europeans, the remainder being derived from Indian personnel. In all other *Salmonella* infections from whom a large number of strains have been received the ratio of European to Indian cases has been very much higher than this. Moreover, the great majority of cases occurred in areas associated with the Burma campaign. Thus, during 1944 and the first two months of 1945, 48·4 per cent of strains were isolated in forward operational areas. 30·7 per cent were received from the District Laboratory, Calcutta, and only 20·9 per cent from areas having no obvious connection with Burma. Although no figures are available, it is understood that infection with *Bact. paratyphosum C* is common among the Chinese and it has been suggested that it may have been introduced into India by Chinese personnel (personal communication—Lieut.-Colonel A. W. D. Leishman, R.A.M.C.). It is also possible that the movements of civilian populations following the Japanese invasion of Burma may have played a part in its introduction. The geographical and temporal distribution of the strains verified by us suggests the increasing frequency of sporadic cases rather than an increasing occurrence of small, localized epidemics. *Bact. paratyphosum C* is said to be particularly prone to localization with pus production. It is worth noting that, of our 102 strains received during 1943 and 1944, five had been isolated from pus, while isolations from this source of *Bact. typhosum* (811 strains), *Bact. paratyphosum A* (166 strains) and *Bact. enteritidis* (105 strains) were all under one per cent.

The problem of *Bact. enteritidis* falls into a rather different category. It has been pointed out earlier that the classical pathogenic rôle of this organism is to cause a localized gastro-enteritis and that accounts of *Bact. enteritidis* septicæmia have been extremely rare in the literature. If this organism had possessed, elsewhere, any degree of invasive function one would expect that the occurrence of septicæmia and septic complications following infection by it would have become widely recognized in view of the large numbers of outbreaks of gastro-enteritis of considerable magnitude which have been reported in detail from Britain and Europe in general and from Western Germany in particular. In fact, such complications have been a rarity, have arisen almost exclusively in the new-born or in those debilitated by age or intercurrent disease, and have superseded rather than masked a gastro-enteritis. Again, *enteritidis* infection usually manifests itself in the form of large or small explosive outbreaks of gastro-enteritis which is rarely fatal, and not in the occurrence of sporadic cases.

The type of *Bact. enteritidis* infection now being encountered in India appears to differ in almost every respect from what has formerly been regarded as typical. From the figures and records that are directly at our disposal the following features stand out:—

1. The type of infection now occurring is a septicæmia with little or no evidence of preceding intestinal involvement. Of our strains 87·5 per cent were isolated from blood and in the brief clinical notes accompanying a proportion of these strains no reference to intestinal localization has been made. Less than four per cent of strains were isolated primarily from the faeces.
2. There appears to be a definite tendency towards localization with pus-formation.
3. The fatality rate is by no means negligible and appears to lie between five and ten per cent.
4. All these cases have occurred in adult males employed in military service.
5. During 1943 there appeared to be no particular regional occurrence of infection, one or two strains being sent now and again from laboratories all over India. Throughout 1944 and the first six weeks of 1945, however, the bulk of strains have been sent from Calcutta, the majority of the remainder being from forward areas. Over the two-year period, 42·0 per cent were isolated in Calcutta. 28·0 per cent in forward operational areas in connection with the Burma campaign, and 30·0 per cent from

areas having no obvious connection with Burma. There has, however, been no indication of any explosive outbreak, the disease appearing, rather, to have been endemic in type. For example, the considerable number of strains from Calcutta were received in ones and twos and threes over a period of more than twelve months.

McDonald (1944) has described three cases of *Bact. enteritidis* septicæmia, one of which was fatal, occurring in Indian troops. This is the first published account of this condition in India. However, in 1943, a report was received by us of the occurrence of an outbreak of invasive *Bact. enteritidis* infection which reached epidemic proportions among Australian troops operating in New Guinea and North Australia. Clinical notes of fourteen patients treated at an Australian general hospital between January and March 1943 were also received (Major A. V. Jackson, A.A.M.C.—Report to the Director of Australian Army Med. Services). From these sources and from information accompanying our own strains a general idea of the clinical picture in such cases may be derived. Cases appear to fall into three main types. In the first there is spiky fever of intermittent type, sometimes accompanied by a few mild bouts of diarrhœa, which persists for from one to two weeks and then settles down. Splenic enlargement is uncommon (if chronic or intercurrent malarial infection is present, as is not uncommon, no conclusions on this point can be drawn), the leucocyte count may be normal, there is no rash and the pulse rate is usually proportional to the increase in temperature. The organism can usually be isolated by blood culture and, occasionally, from the stools. The appearances are those of an uncomplicated septicæmia. The second variety of case is characterized by long continued pyrexia of septicæmic type which may persist for up to eight weeks. Enlargement of the spleen may be present but there is no rash. Leucopenia and relative bradycardia may or may not be present. After a variable period of uncomplicated fever, localization with pus-production sets in. Abscess-formation in the subcutaneous tissues, periostitis, empyema, meningitis or cystitis may be found. Jackson's series is remarkable for the number of cases developing infection of the urino-genital system. McDonald mentions the occurrence of a certain amount of abdominal pain and chest symptoms, though, in the one fatal case in his series of three, no obvious intestinal involvement was apparent at post mortem apart from prominence of the lymph follicles. The third type of case displays the full clinical picture of enteric fever with continuous pyrexia, splenomegaly, rose spots, leucopenia and relative bradycardia. There is a tendency here, too, to localization with pus-production—not a common feature of *Bact. typhosum* infection. The Indian cases appear to be more of the pure septicæmic variety than of enteric type though it is not improbable that some of these latter have been wrongly diagnosed as *Bact. typhosum* infections, especially as the Widal reaction may show a rising titre against *Bact. typhosum* (O) antigen. The two cases in McDonald's series on whom successive Widal reactions were performed showed a rising *Bact. typhosum* titre but only in one did the titre rise above 320. In neither case was a significant *Bact. enteritidis* (H) titre present. The majority of Jackson's cases showed *Bact. enteritidis* titres of 250 or over but he does not state whether (H) or (O) type agglutination was employed. (H) type agglutination might be expected to be of value in the diagnosis of *Bact. enteritidis* infection since, in this case, an anamnestic rise in titre should not be conditioned by T. A. B. prophylaxis. The only certain method of diagnosis, however, lies in isolation of the causative organism either by blood culture or from urine, pus or fæces. Blood culture appears to be by far the most certain method and it is important to realize in this connection that, certainly in the septicæmic type of case, it may be successful throughout the course of the pyrexia. It must be made clear that the clinical types described have only arbitrarily been made clear cut for purposes of description and that there may be considerable overlap in the clinical picture of any one case.

The mode of spread of this variety of *Bact. enteritidis* infection is not known. McDonald suggests, by analogy from the common ætiology of *dublin* infection in Denmark and South Africa and the fact that the only common factor preceding infection in his three cases was a journey on the Indian railways, that spread may

be by infected milk. This does not seem likely, however, in the majority of cases in view of the newness of the clinical type of the disease and its widespread occurrence. The incidence of human *Salmonella* disease of which an animal is the reservoir is usually determined by the animal distribution of the type concerned and the food habits of the people. As an example of this, Kauffmann (quoted by Bornstein, *loc. cit.*) has attributed the high incidence of *Bact. enteritidis* infection in Western Germany to the consumption of ducks' eggs imported from Holland where the infection rate of this organism in ducks is high. Again, human infection from *Bact. enteritidis* var. *dublin* in South Africa is associated with the enzooticity of this type in calves and cattle. In Europe, outside Denmark, *Bact. enteritidis* and *Bact. typhimurium* replace the *dublin* variant in calves and *dublin* infection in man is correspondingly rare. It is unlikely that *gartner* infection of cattle could have become so widespread in India in so short a time or that the food habits of military personnel have undergone any radical change. There is no doubt, however, that the richest reservoir of *Salmonella* disease in general and of *Bact. enteritidis* infection in particular is the animal carrier. In Europe and North and South America, rodents, fowl, swine and cattle have been found to harbour the organism. In India, natural epizootics among laboratory animals is not uncommon. It is, therefore, possible that the consumption of infected food is the primary aetiological factor. It is most improbable that this factor is the eating of infected meat in view of the high incidence of the disease in Hindus. A survey of the incidence and type of *Salmonella* infection in the common animals of India is long overdue. The change in the pathogenic rôle of *Bact. enteritidis* remains unexplained. An alternative theory is that some of the diarrhoeas of unknown aetiology which are so prevalent in this country have been, in fact, mild, localized *gartner* infections resulting from contamination of food or water by an animal reservoir and that these infections have become apparent only through the adoption by the organism of more invasive attributes. There are three ways in which the virulence of *Bact. enteritidis* may have become enhanced :—

1. By the acquisition by mutation of a 'virulence' antigen analogous to the Vi antigen of *Bact. typhosum* ;
2. By a biological variation of unknown origin associated with an increased tendency towards invasiveness, such as is exemplified by the cyclic variation, with a periodicity of about 30 years, in the severity of scarlet fever and the incidence of severe septic complications in that condition, or
3. By the development of a high degree of host-adaptation following the replacement of the animal reservoir by the human carrier as the normal source of infection.

It is indeed strange that two organisms so closely resembling one another as *Bact. typhosum* and *Bact. enteritidis* in the structure of their somatic antigen should typically give rise to such different types of disease, for pure 'W' forms of the former bacterium have undoubtedly been responsible for cases of true enteric fever. In fact, it is easier to understand the prevalent invasiveness of *Bact. enteritidis* than it is to explain its usual rôle in causing only a gastro-enteritis. The fundamental difference between the two conditions lies, of course, in the mode of spread and, in the present state of our knowledge, the development by *Bact. enteritidis* of host-adaptation to man by human passage appears to supply at least a plausible explanation. On the other hand, careful antigenic analysis of strains isolated from simple gastro-enteritis and from septicæmic and 'enteric' cases may throw further light on the problem.

In view of the findings presented in this paper, the advisability of including in the vaccine in common use for the prophylaxis of the enteric group of fevers in India, the elements necessary to cover *Bact. paratyphosum* C and *Bact. enteritidis* is worthy of consideration.

Bornstein (*loc. cit.*) has postulated that the old terms relating to *Salmonella* infection in man should be given up in the light of modern knowledge of the diversity

of ways in which almost any *Salmonella* may manifest itself, and should be replaced by a new and more comprehensive nomenclature. He suggests the following terminology:—

1. *Salmonella fever*.—This condition is synonymous with 'enteric fever' of all degrees of severity or mildness but the term recognizes the fact that *Salmonella* other than *Bact. typhosum* and *Bact. paratyphosum A* and *B* may be causative of the condition.
2. *Salmonella septicæmia*.—This is a condition similar in its clinical manifestations to septicæmia due to pyogenic cocci but caused by organisms of the *Salmonella* group. It embraces uncomplicated cases as well as those in whom localization with pus-production occurs and the descriptions of *gartner* septicæmia given above may be regarded as characteristic of the group.
3. *Salmonella gastro-enteritis*.—Our findings and especially the high incidence of both common and uncommon *Salmonellas* derived from blood culture or other source indicative of invasion afford strong support for Bornstein's suggestion.

The most important recent advances in our knowledge of the *Salmonella* group are the realization of two facts. The first is that, with the possible exception of *Bact. typhosum*, almost any member is capable of giving rise to any of the above clinical types of disease in man although certain have a predilection for one rather than the other types. The second is that, again with the exception of *Bact. typhosum*, and, probably, *Bact. paratyphosum C*, any member of the group may be found in and be spread by animals and that host-adaptation may profoundly affect the course of human disease.

SUMMARY.

1. A survey of *Salmonella* types responsible for disease in the Army in India from 1941 to the beginning of 1945 has been made.
2. Attention has been drawn to the marked increase in the number of *Bact. paratyphosum C* and *Bact. enteritidis* infections reported in recent years.
3. By far the greater proportion of *Bact. enteritidis* strains have been isolated by blood culture or from anatomical sources which imply for the organism an invasive rôle in man.
4. Possible reasons for the apparent increase in *Bact. paratyphosum C* and *Bact. enteritidis* infection are discussed.
5. The clinical types of invasive *Bact. enteritidis* disease are described.
6. An account is given of interesting or instructive findings in connection with the bacteriology of the *Salmonella* group.

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STUDIES IN CALCIUM AND PHOSPHORUS METABOLISM.

Part VII.

THE IONIC PRODUCTS OF CALCIUM PHOSPHATES IN EXPERIMENTALLY INDUCED VITAMIN D DEFICIENCY.

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IN an earlier paper (Patwardhan, Chitre and Sukhatankar, 1944) experimental evidence has been presented to show that the serum of infants and children suffering from incipient or active rickets was undersaturated, according to current concepts (Logan, 1940) with respect to the salts CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$. The serum of children not suffering from rickets indicated, on the other hand, a state of saturation with respect to both these salts. Similar results have been obtained from the study of induced rickets in puppies, a short note on which has already been published (Patwardhan and Sukhatankar, 1943). The full details of the experiments are reported in the present paper.

EXPERIMENTAL.

Young puppies of mongrel breed from one or two litters were used for the experiments which were repeated three times in all with fresh batches of puppies. The animals were put on experimental diets when they were about five to six weeks old, the litter mates being distributed equally between the control and the rachitic groups. The composition of the diets is given in Table I :—

TABLE I.

Composition of the diets.

Article.	Diet I.	Diet II.
1. Rice	220.0 g.	220.0 g.
2. White bread	57.6 g.	57.0 g.
3. Skimmed milk, dried	7.5 g.	...
4. „ „ liquid	153.0 c.c.	...
5. Egg-white, dry	8.0 g.
6. Sodium chloride	1.0 g.	...
7. P-free salt mixture	5.0 g.
8. Marmite	1.0 g.	...
9. Yeast	5.0 g.
10. Red-palm oil	1.0 g.	1.0 g.
11. Ascorbic acid	10.0 mg.	10.0 mg.

The phosphorus-free salt mixture used in diet II was prepared according to Freeman and McLean (1941). The various articles of diet (1 to 9) were mixed, cooked with water and fed *ad lib.* Water was also given *ad lib.* Red-palm oil, vitamin D in oil (to controls only) and ascorbic acid in solution were fed directly once a week in weekly doses.

The experiments were begun in the month of June just prior to the onset of the monsoon, and they lasted from 16 to 31 weeks. During the first 16 weeks, which constitutes the rainy season in Bombay, sunlight was at the minimum and during the cloudy periods, which were quite frequent, the dogs could be left in the open air for exercise.

Blood was taken from each dog once a fortnight. The method of blood collection depended upon the analytical procedure to be adopted, e.g. for determinations of pH it was collected under paraffin and delivered into tubes under a layer of paraffin. For other analyses the blood was allowed to clot, the serum removed by centrifuging and the following determinations carried out on the serum: (1) total calcium, (2) inorganic and total acid-soluble phosphorus (methods are described in the earlier papers in this series), and (3) total protein by Kjeldahl's method. In addition to these, magnesium, albumin, globulin, non-protein nitrogen, chlorides and total base in the serum were also estimated by suitable methods. The results of these latter showed absence of correlation between the state of vitamin D deficiency and their concentration in the serum. Hence the results are not included in this paper. The pH of the plasma was determined by the method of Hastings and Sendroy (1924) on the blood drawn for the purpose as already stated.

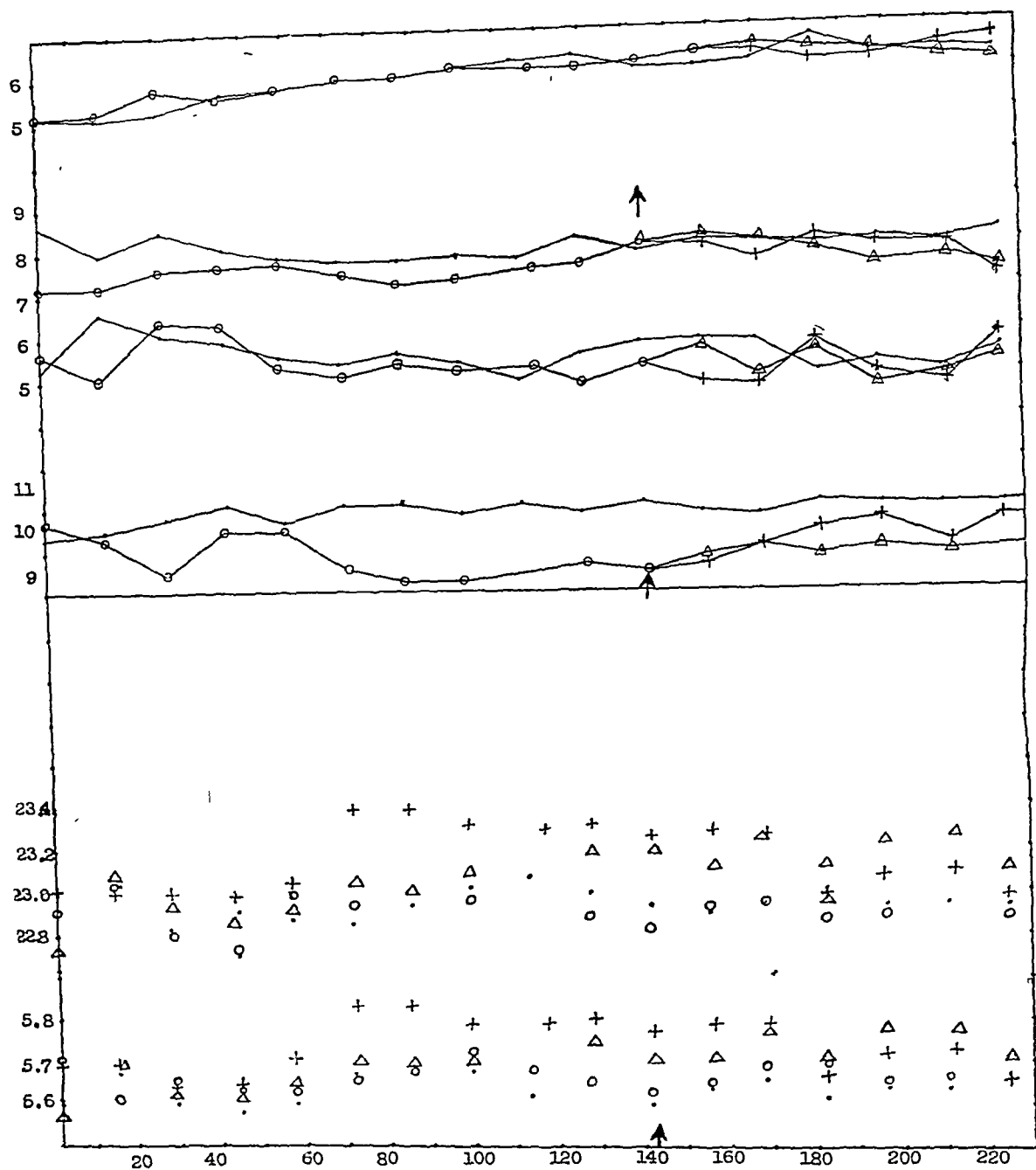
Once a fortnight the distal ends of the radius and ulna were subjected to radiological examination. Nearly a month after definite radiological evidence of rickets was obtained the administration of vitamin D was begun. In the first and second experiments the dogs received diet I. The animals in the third experiment were started on diet I, and later, in the 9th week of the experiment, the dogs were put on diet II which resembled in some respects the one given by Freeman and McLean (*loc. cit.*) to their experimental dogs. On this diet the rickets was more severe than on diet I. On the whole diet I was a good diet being deficient only in vitamin D. The development of rickets in pups fed this diet could be considered analogous to the rachitic process in infants and children under clinical conditions. This, however, cannot be said to be the case in pups fed diet II. Graphs 1 and 2 show the typical results in the control and rachitic dogs of the 2nd and 3rd batch. The observations on the 1st batch were similar to those on the 2nd batch. They have, therefore, been excluded merely to avoid repetition. The 3rd batch of dogs is included because the new rachitogenic diet (diet II) brought out certain trends in the total calcium and inorganic phosphorus of the serum which were dissimilar to those observed in the earlier experiments.

DISCUSSION.

Experiments with the second batch of puppies.

In Graph 1 are plotted the data for calcium, inorganic and total acid-soluble phosphorus and the total protein of the serum of the dogs of the 2nd batch, from the beginning to the termination of the experiments. The results are discussed separately under each heading.

Calcium.—The calcium content of serum of the two dogs on vitamin D deficient diet progressively decreased as the experiment proceeded, the values being lower than that of the controls by about 1.5 mg. per 100 c.c. Till the time when vitamin D administration to dog 14 was started the value for calcium remained almost constant between 8.75 mg. and 9.00 mg. per 100 c.c. serum as against 11.00 mg. per 100 c.c. in the control animals during the same period. On administration of vitamin D (4,000 I. U. per day) the calcium content of the serum of dog 14 rose gradually, while that of dog 12, which did not receive vitamin D, was also rising in the meanwhile but the level was always lower than that of dog 14.



GRAPH 1.—Experiments on dogs 7 (●), 11 (○), 12 (△) and 14 (+)

↑ Vitamin D started.

Upper four graphs: Controls 7 and 11 .—.

Vitamin D deficient 12 and 14 to the day of vitamin D administration o—o
afterwards dog 12 △—△, dog 14 +—+

Total protein.—The total protein of both the groups of dogs ran close to each other. The effect of vitamin D on the protein content of the serum of dog 14 was not particularly noticeable.

Serum inorganic phosphorus.—The serum inorganic phosphorus of the vitamin D deficient dogs was a little lower than that of the controls but rose slightly in dog 14 after vitamin D administration and was on an average about 0.5 mg. higher than the serum inorganic phosphorus of dog 12.

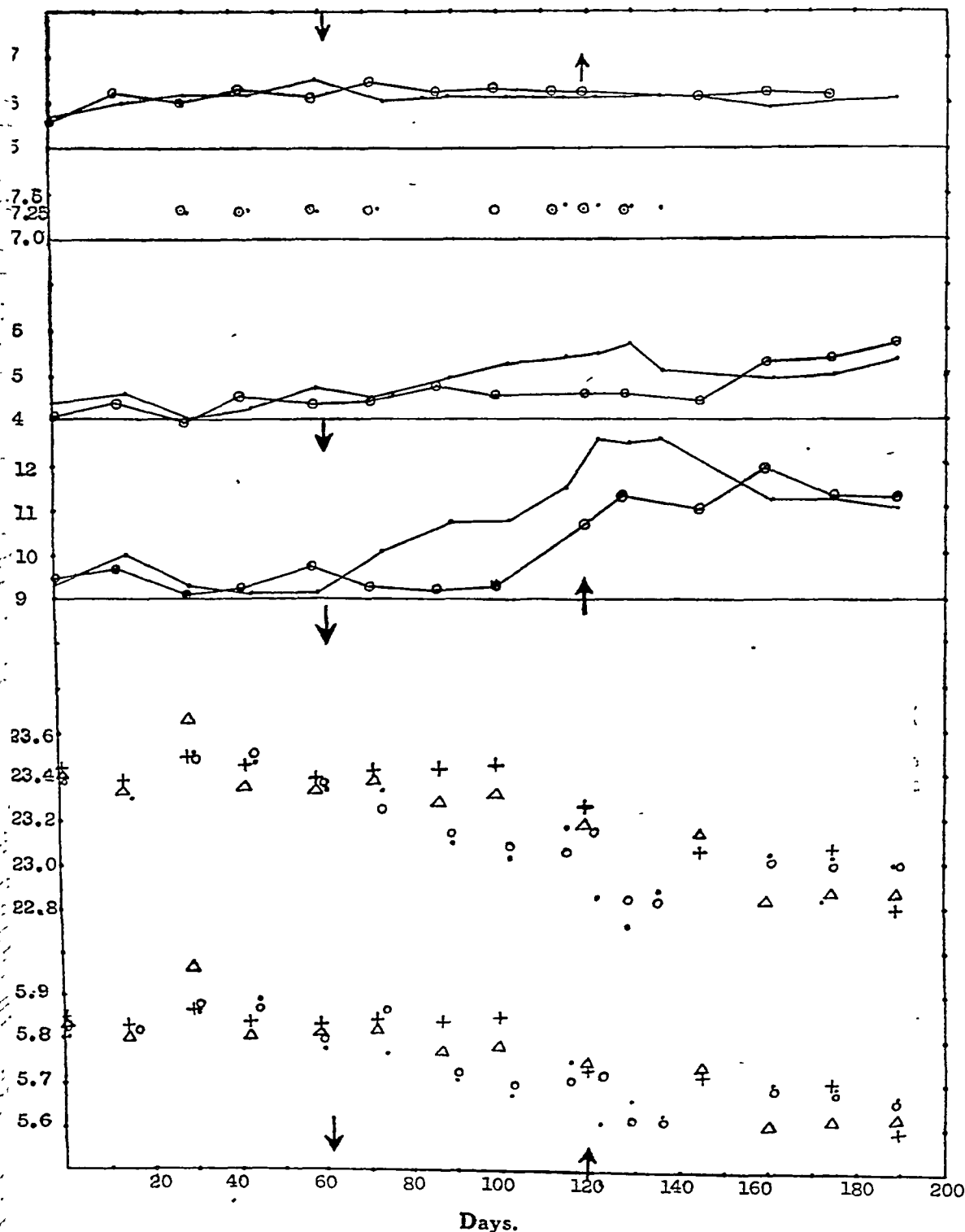
Serum acid-soluble phosphorus.—The behaviour of the total acid-soluble phosphorus was irregular. In vitamin D deficient dogs the values were lower right from the beginning, but it is difficult to say, however, whether the approximation in the values for rachitic and control animals from the time of vitamin D administration was due to the supplement of vitamin D or otherwise. The evidence is not conclusive.

The ionic products.—The fact that serum calcium in vitamin D deficient dogs decreased while the protein content was slowly but steadily increasing had an adverse influence on the $[Ca^{++}]$ of their serum, while that of the serum of control animals was not similarly affected because in the beginning the total calcium and total protein both showed a slight increase. From 43rd day onwards the calcium remained more or less constant, while the rate of protein increase was considerably slowed down. Attempts were made to determine biologically the $[Ca^{++}]$ in dog serum but owing to the presence of a pressor substance in it, as previously reported by McLean and Hastings (1935), the method was unsuccessful; the value was therefore estimated by reference to the nomogram worked out by these authors by the application of the Mass Law equation to protein and total calcium of blood serum. The values of $[HPO_4^{=}]$ and $[PO_4^{=}]$ at pH 7.4 were calculated from the total PO_4 using the formulæ of Sendroy and Hastings (1926). In Graph 1 are given the values for pK s.p. $CaHPO_4$ and pK s.p. $Ca_3(PO_4)_2$. The significance of these findings is discussed further on.

Experiments with the third batch of puppies.

Four puppies from one litter were divided into two groups: Dogs 15 and 16 served as controls; dogs 17 and 18 received the vitamin D deficient diet. Both the groups were started on diet I, the control animals receiving a supplement of 500 I. U. of vitamin D per dog per day in weekly doses. On the 61st day of the experiment the dogs were put on diet III. Radiologically mild rickets could be made out in all the dogs of this group even at the beginning of the experiment. This might probably have been due to the poor dietetic history of the mother dog, and the subsequent poor nutrition of the puppies before they were obtained for the experiment. The change over to diet II which was severely rachitogenic did not materially alter in the beginning the radiological picture of the control dogs. On the other hand with the progress of the experiment it improved resulting in the elimination of the slight epiphyseal gap which had previously existed in the control animals. Rickets in dog 17 was mild but in dog 18 it was florid. On the 119th day of the experiment the administration of 4,000 I. U. of vitamin D per day per dog to dogs 17 and 18 was commenced. The x-ray picture of the distal end of radius and ulna showed recovery after 30 days.

Calcium.—As all the four animals were probably slightly rachitic from the beginning the difference in the serum calcium of the two groups is not marked (Graph 2). It becomes so, however, after the change over to diet II. Rise in serum calcium is observed in both the groups but it is much greater in the control than in the rachitic group. This rise is later followed by a fall in the control group, a fact which had been observed earlier by Freeman and McLean (*loc. cit.*) in their pups on a somewhat similar diet. It is difficult to decide whether the administration of vitamin D to dogs 17 and 18 had any influence on the changes in the concentration of serum calcium in this



GRAPH 2.—Experiments with dog. 15 (●), 16 (○), 17 (△) and 18 (+)
 ↓ Change to diet II. ↑ Vitamin D started.

particular experiment. At the time of recovery the values in both groups were approximately the same.

Total protein.—The behaviour of total protein of serum was very similar to the one already described in the previous experiment. The change over to diet II did not seem to influence the total protein of the serum.

pH.—The plasma pH of both groups ran closely parallel and was unaffected by the severity of rickets, the change over from one diet to the other, or even by the administration of vitamin D to rachitic dogs.

Inorganic phosphorus.—The serum inorganic phosphorus was very much at the same level in both groups till after the change over to diet II. After that it increased in the control group but remained unaffected in the rachitic dogs. Even after vitamin D was administered to dogs 17 and 18 the serum inorganic P did not show a rise for nearly 30 days; after that it rose to a higher level than that of the control dogs.

The ionic products.—The negative logarithm of the ionic products for CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$ probably indicates a state of unsaturation in the serum of puppies in the beginning. These products did not differ very much among the four animals till the day of the alteration in the diet. After that the values for the control dogs were near the points indicating saturation, while those for the rachitic dogs indicated undersaturation. On administration of vitamin D these latter moved close to the controls and at the end of the experiment in both the groups the serum appeared to be saturated with regard to CaHPO_4 . The healing process set in after vitamin D was administered and appeared radiologically to be fairly well advanced within 30 days, but the movement of the ionic product towards the saturation point was slower, a fact which it is rather difficult to explain.

The significance of the changes observed in the solubility products of $[\text{Ca}^{++}]$ $[\text{HPO}_4^{--}]$ and $[\text{Ca}^{++}]^3 \times [\text{PO}_4^{--}]^2$ with the progress and cure of the vitamin D deficiency state is not yet sufficiently clear. For the sake of clarity the relation between these two products and calcification will be discussed separately. Wendt and Clarke (1923) first suggested that formation of CaHPO_4 was the first step in the deposition of bone-salt. Later, Shear and Kramer (1928) observed that the serum of rachitic infants was definitely undersaturated with respect to CaHPO_4 and that even the normal serum was slightly undersaturated. Freeman and McLean (*loc. cit.*) in their experiments with puppies, found a correlation between $\text{pK s.p. } [\text{Ca}^{++}] [\text{HPO}_4^{--}]$ and the state of calcification, although no such relation could be shown to exist with respect to $\text{Ca}_3(\text{PO}_4)_2$. Patwardhan, Chitre and Sukhatankar (*loc. cit.*) have found well-defined differences in $\text{pK s.p. } [\text{Ca}^{++}] [\text{HPO}_4^{--}]$ between the serum of rachitic and non-rachitic infants and children. The experiments described in the present paper also show that vitamin D influences the solubility product of $[\text{Ca}^{++}] [\text{HPO}_4^{--}]$. In vitamin D deficiency the serum appeared to be undersaturated with respect to CaHPO_4 , a state which was altered by administration of vitamin D. Even before vitamin D deficiency had progressed so far as to render the diagnosis of rickets radiologically positive the serum was undersaturated and during the course of healing the solubility products moved back towards the point of saturation.

The correlation between the state of vitamin D deficiency and the solubility product of $[\text{Ca}^{++}] [\text{HPO}_4^{--}]$ thus appears to support the view that precipitation of CaHPO_4 is probably the first step in the formation of bone-salt as explained by Logan (*loc. cit.*). That the salt thus formed immediately undergoes modification by exchange of ions CO_3 , OH , PO_4 , etc., causing a change in the mineral composition of bone is a hypothesis which receives indirect support from the observations on the inconstant composition of bone (Kramer and Shear, 1928; Patwardhan and Chitre, 1938).

From the experiments reported here it appears that pK s.p. values of $CaHPO_4$ could be used as a test indicating a state of vitamin D deficiency in general rather than any particular stage of the rachitic process, a finding which is in agreement with the observation made in the study of clinical rickets when no correlation could be shown between the solubility product and the degree of rickets (Patwardhan, 1943).

Certain observations based on physico-chemical considerations and reported from other laboratories during the last two or three years seem to challenge the significance of such results. Ludewig, Chanutin and Masket (1942) question the reliability of McLean and Hastings' (1935a) procedure of applying Mass Law equation to serum for determination of serum $[Ca^{++}]$. The accuracy of the claim made by Logan and Taylor (1937) 'that the ion product $[Ca^{++}]^3 \times [PO_4^{==}]^2$ increases as the amount of bone or tricalcium phosphate, equilibrated with solution of their ions, decreases below 150 mg. per litre' has also been challenged by Greenwald (1942). He believes that their data are more in accord with the view that mixtures containing smaller amounts of precipitate were supersaturated and suggests that an apparently true equilibrium constant pK s.p. = approximately 27 at $38^\circ C$. is attained with large amounts of precipitate because with the large surface offered, equilibrium at the surface is more readily attained. In a later communication Greenwald (1942a) proposes to regard $Ca_3(PO_4)_2$ as the calcium salt of the acid $CaHPO_4$ which renders more reasonable the rapidity of precipitation and the slowness with which equilibrium is attained and the increasing content of Ca , OH or CO_3 in the precipitate as the pH is increased and suggests the use of the solubility product $[Ca^{++}] [CaPO_4^-]^2$. Eisenberger *et al.* (1940) point out that the solubility product of bone-salt cannot be expressed merely by $[Ca^{++}]^3 \times [PO_4^{==}]^2$ as the concept is meaningless without reference to a known solid phase of comparatively simple ionic composition. Thus, there are two uncertainties—(1) the composition of the bone-salt in equilibrium with electrolytes of the serum, and (2) the ionic product of $[Ca^{++}]^3 \times [PO_4^{==}]^2$ at which equilibrium is presumably attained. If the claim of Greenwald is conceded one has to accept that serum of normal as well as rachitic infants is supersaturated with respect to $Ca_3(PO_4)_2$ and the changes in pK s.p. $[Ca^{++}]^3 \times [PO_4^{==}]^2$ such as have been observed in clinical and experimental rickets have no meaning. McLean (1943) points out, however, that the local calcification mechanism in rachitic cartilage is active in solutions varying over a range of pH 5.7 to 8.7. At pH 7.0 and higher the critical point for calcification is correlated closely with the solubility product $[Ca^{++}] [HPO_4^{==}]$. On the acid side of pH 7.0 the correlation is with $[Ca^{++}]^3 \times [PO_4^{==}]^2$ of Logan and Taylor (*loc. cit.*). The curves of two products cross at pH 7.0 and the generalization may be made that both products must be exceeded for calcification to occur.

Thus it appears that there is some confusion in the opinions concerning the correlation between the ionic products of calcium phosphates and process of calcification with the result that no one hypotheses would quite fit the facts. One further difficulty in the way of accepting this hypothesis as it stands is that the serum of normal healthy adults is always undersaturated with regard to $CaHPO_4$ although the position regarding $Ca_3(PO_4)_2$ is now uncertain. Further work is obviously necessary and the correct interpretation of observed facts will have to await the elucidation of these points.

SUMMARY.

Vitamin D deficiency has been induced in dogs on two types of diets, one a diet deficient only in respect of vitamin D and the second a diet deficient not only in vitamin D

but in which the phosphorus content was very low. The serum calcium, serum inorganic and acid-soluble phosphorus, serum protein, and plasma pH (in the 3rd batch of experiments) were determined at fortnightly intervals throughout an experimental period lasting 16 to 31 weeks. The findings on these two diets are summarized separately.

(1) *On diet I.*—Serum calcium decreased as vitamin D deficiency increased; serum inorganic phosphorus as compared with the controls was only slightly reduced. On administration of vitamin D, the serum calcium rose earlier than the inorganic phosphorus. No significant change in the total acid-soluble phosphorus of the serum was observed. The protein content of serum slowly increased with age in both the control and the rachitic groups; vitamin D administration had no influence on the total serum protein of the rachitic dogs. The values for pK s.p. for CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$ increased as the deficiency of vitamin D progressed, and decreased after the administration of vitamin D.

(2) *On low phosphorus diet.*—The young puppies used for this experiment showed a mild degree of rickets even at the beginning of the experiment. They were started on the same diet as in (1) but were later changed over to a low phosphorus diet. After this change hyper-calcæmia set in in both the control and the rachitic groups. It set in earlier, however, in the control dogs, declining somewhat later on. The serum inorganic phosphorus was lower in the rachitic group and rose slowly after administration of vitamin D. The concentration of serum protein was unaffected by (a) the change in diet, (b) the onset of severe rickets, or (c) the administration of vitamin D. The plasma pH in both groups also remained unchanged throughout the experiment. At the beginning of the experiment the values for pK s.p. CaHPO_4 indicated undersaturation in all the four dogs. In control dogs receiving 500 I. U. vitamin D per day per dog they approached the saturation point whereas those of rachitic dogs moved slowly towards saturation even after the administration of 4,000 I. U. vitamin D per day per dog.

The observation made in clinical rickets that the ionic products of calcium phosphates are influenced by state of vitamin D nutrition has been confirmed.

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STUDIES ON THE DESTRUCTION OF VITAMIN A IN SHARK-LIVER OIL.

Part V.

EFFECT OF SOME ANTI-OXIDANTS ON THE STABILIZATION OF VITAMIN A.

BY

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SHARK-LIVER OIL is a rich source of vitamin A in India. The average vitamin A potency of Indian shark-liver oils is much higher than that of cod-liver oils. Most of the oils manufactured at present in India, however, have been reported to lose their vitamin A rapidly on storage (Dattatreya Rao, 1944). Hence, a study of the methods of preservation of vitamin A in shark-liver oil is of considerable importance to this growing industry.

The development of rancidity and the destruction of vitamin A in fats and oils have been reported to proceed simultaneously (Fridericia, 1924; Powick, 1925; Rosenheim and Webster, 1926; Wokes and Willimott, 1927; Whipple, 1934; Smith, 1939). The chief cause of rancidity and destruction of vitamin A in fats and oils is oxidation (Brocklesby, 1941).

The keeping quality of oils is greatly influenced by their content of natural anti-oxidants which are oxidized in preference to the glycerides (Mattill, 1931). Since Moureu and Dufraisse (1922) reported that hydroquinone can increase the stability of oils, a large number of chemicals having similar anti-oxidative properties have been found. In this paper, the comparative inhibiting property of a number of such compounds has been studied with reference to shark-liver oil.

The chemical anti-oxidants can be broadly classified under the following heads:—

(1) *Acid-type*.—Inorganic and organic acids like sulphuric, ortho-phosphoric, citric, oxalic, etc., and their salts have anti-oxidative effects on fats and oils (Olcott and Mattill, 1936). Acid-type inhibitors, however, have been reported to lose their efficiency as the unsaturation of the fats increases (Brocklesby, *loc. cit.*).

(2) *Phenolic*.—A number of phenolic compounds have proved to be effective inhibitors, particularly hydroquinone (Olcott, 1934), pyrogallol (Wagner and Brier, 1931), pyrocatechol (Olcott, *loc. cit.*), α - and β -naphthol (Mattill, *loc. cit.*), resorcinol (Wagner and Brier, *loc. cit.*), guaiacol (Coe and Leclerc, 1935), 1:5 dihydroxy-naphthalene (Lea, 1944), pure tocopherols and their extracts (Golumbic, 1943; Hilditch, 1944). Gallic acid and its esters have been shown to afford marked protection to both animal and vegetable oils (Golumbic and Mattill, 1942). Boehm and Williams (1943) reported the inhibiting action of propyl gallate on lard and cod-liver oil mixture. Hilditch (*loc. cit.*) has recommended the use of gallates, specially ethyl gallate, as anti-oxidants in various foodstuffs. Lea (1944) has shown that even 0.01 per cent of ethyl gallate is very effective in delaying the onset of tallowiness in lard. Nair and Ramakrishnan (1944) studied the effect of hydroquinone, gallic acid, etc., on shark-liver oil.

(3) *Nitrogenous*.—Various organic nitrogen compounds including amines (Wagner and Brier, *loc. cit.*) and amino-acids like glycine, glutamic and aspartic acids (Lea, 1936) also possess inhibiting properties.

Apart from the chemical anti-oxidants, natural inhibitors, occurring in cereal flours and their extracts (Bradway and Mattill, 1934; French, Olcott and Mattill, 1935; Peters and Musher, 1937) and the crushed oil-bearing seeds (Musher, 1935), can be concentrated and added to the oil to increase the stability. Among the cereal flours, oat flour has been very effective on halibut-liver and salmon oils (Lowen *et al.*, 1937). In this laboratory, it was shown that Kamala dye inhibits the oxidative deterioration of ghee (Govindarajan and Banerjee, 1939).

In spite of the large number of anti-oxidants reported in the literature, relatively few meet all the requirements for use in foodstuffs. In addition to inhibiting action, an anti-oxidant must possess the following properties to be safely used in foodstuffs: (1) It must be non-toxic and pharmacologically safe. (2) It should be sufficiently soluble or dispersible in the oil to which it is added. (3) It should not affect the normal taste, colour and flavour of the oil. (4) It should be nearly neutral in reaction. (5) Lastly, it should be cheap. The toxicity of many powerful chemical anti-oxidants prevents their use in edible products and limits the number of possible materials that may be used.

EXPERIMENTAL.

Since oxidation is the main cause of development of rancidity in oils, a suitable method for determining the relative stabilities of different oils, or of the same oil treated in various ways, is to follow the absorption of oxygen under identical conditions. Oxygen-absorption tests, however, are time-consuming unless a certain amount of acceleration to promote oxidative changes is allowed. So, to test the effectiveness of the anti-oxidants within a reasonable length of time, the method of determining the length of 'induction period' from oxygen absorption as described by Greenbank and Holm (1925) and modified by Govindarajan and Banerjee (1940) has been followed. The method depends principally on the measurement of oxygen absorbed by a thin layer of oil, maintained at a high temperature and exposed to oxygen in a closed conical flask, by means of pressure changes recorded on a mercury manometer attached to the flask. The suitable high temperature for routine experiments was determined by measuring the induction periods at three different temperatures, viz. 55°C., 65°C. and 75°C., and it was observed that at 75°C. the induction period of the control oil was reduced to a short measurable period.

A sample of oil of acid value 0.52 was used in all the experiments. Most of the acid-type and some of the phenolic inhibitors and a few of the amino-acids were tried. Requisite amount of anti-oxidant was weighed and incorporated with the oil by trituration in a mortar, as most of the anti-oxidants used did not dissolve in the oil completely. Boehm and Williams (*loc. cit.*) have shown that the trituration of an anti-oxidant in a mortar effectively introduces it in the oil.

TABLE I.
Protection afforded by single anti-oxidants at 75°C.

Anti-oxidants added and concentration (per cent).					Induction period in hours.	Protection factor.
Control	1	...
Tartaric acid (0.1)	1	0
Citric acid (0.1)	1.5	0.5

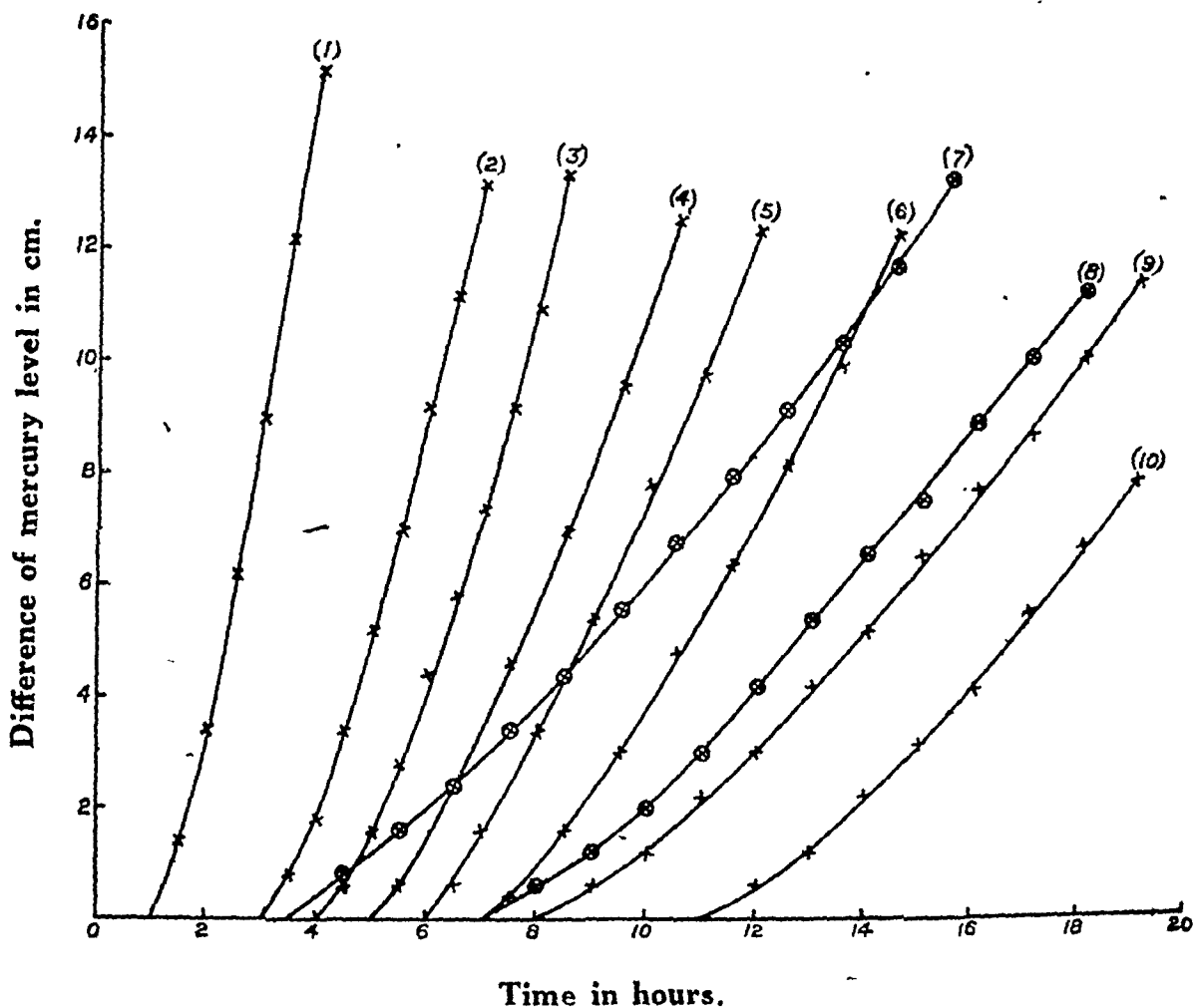
TABLE I—*concl'd.*

Anti-oxidants added and concentration (per cent).				Induction period in hours.	Protection factor.
Malonic acid (0.1)	1.5	0.5
Maleic acid (0.1)	1	0
Succinic acid (0.1)	1	0
Oxalic acid (0.1)	1.5	0.5
Ortho-phosphoric acid (0.1)	3	2
Glycine (0.1)	1	0
Glutamic acid (0.1)	1	0
Tannic acid (0.1)	1	0
α -naphthol (0.1)	1	0
β -naphthol (0.1)	1	0
Resorcinol (0.1)	1	0
Pyrocatechol (0.1)	1.5	0.5
Phloroglucinol (0.1)	1.5	0.5
Hydroquinone (0.1)	3.5	2.5
Kamala dye (0.1)	2	1
Gallic acid (0.1)	4	3
Methyl gallate (0.1)	5	4
Ethyl gallate (0.1)	8	7
" " (0.05)	5	4
Propyl gallate (0.1)	7	6
" " (0.05)	4	3
Iso-butyl gallate (0.1)	11	10
" " (0.05)	6	5
Iso-amyl " (0.1)	7	6
" " (0.05)	4.5	3.5

The lengths of the induction periods were determined from the curves drawn with time in hours as abscissæ and the difference of mercury levels as ordinates. The 'protection factor' was calculated according to the recent method adopted by Hilditch (*loc. cit.*) and

GRAPH 1.

Effect of single anti-oxidants at 75°C.



- (1) Control oil.
- (2) Ortho-phosphoric acid (0.1%).
- (3) Gallic acid (0.1%).
- (4) Methyl gallate (0.1%).
- (5) Iso-butyl gallate (0.05%).
- (6) Propyl gallate (0.1%).
- (7) Hydroquinone (0.1%).
- (8) Iso-amyl gallate (0.1%).
- (9) Ethyl gallate (0.1%).
- (10) Iso-butyl gallate (0.1%).

Lea (1944); it is the ratio of the induction period of the oil treated with anti-oxidant to that the untreated oil minus one. The results are summarized in Table I and some of the important curves are shown in Graph 1.

TABLE II.

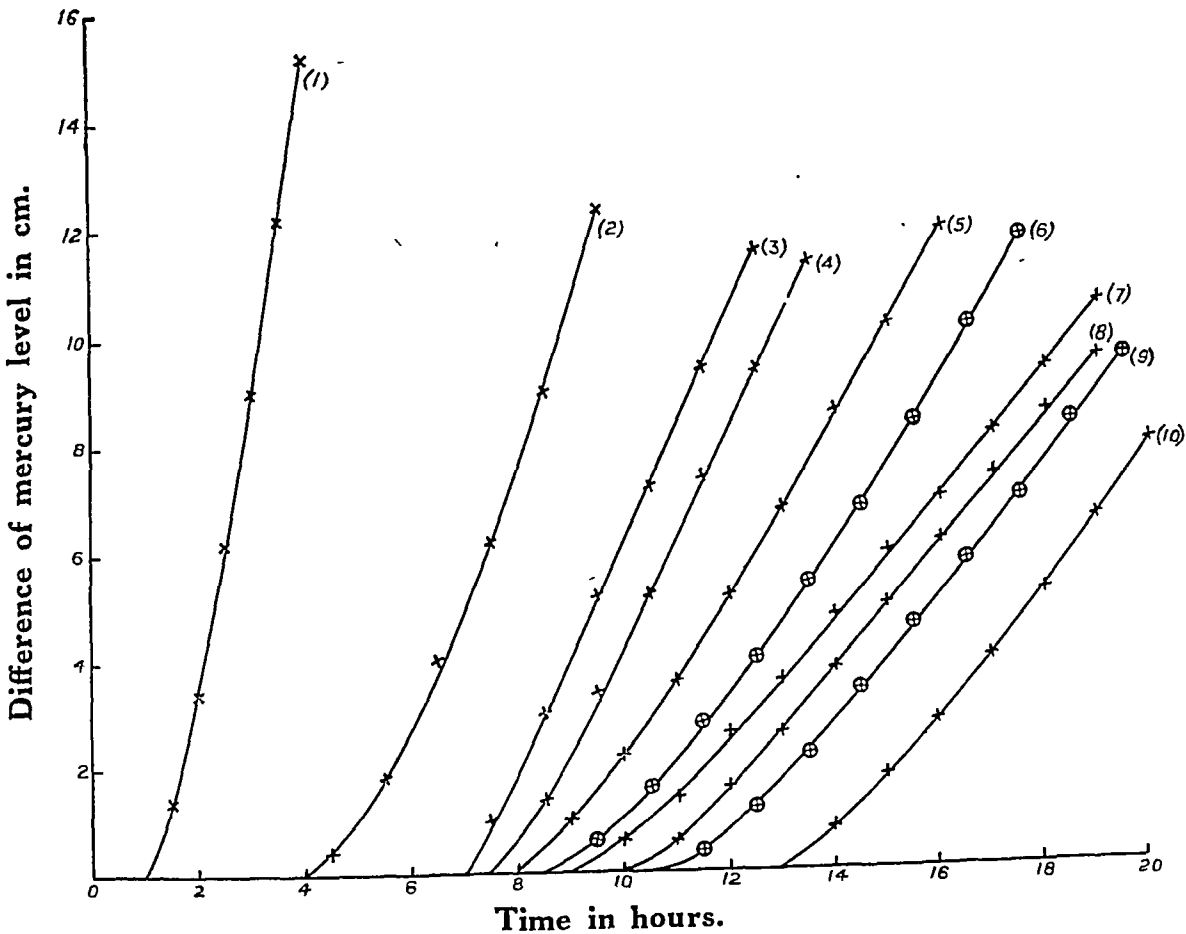
Synergetic effect of mixtures of anti-oxidants at 75°C.

Anti-oxidants added and concentration (per cent).					Induction period in hours.	Protection factor.
Control	1	...
Iso-butyl gallate (0.05) +	ortho-phosphoric acid	(0.05)			13	12
Ethyl gallate (0.05) +	"	"	(0.05)		9.5	8.5
Iso-amyl gallate (0.05) +	"	"	(0.05)		7	6
Propyl gallate (0.05) +	"	"	(0.05)		6	5
Iso-butyl gallate (0.05) +	citric acid	(0.05)		...	10.5	9.5
Ethyl gallate (0.05) +	"	"	(0.05)	...	7	6
Iso-amyl gallate (0.05) +	"	"	(0.05)	...	5	4
Propyl gallate (0.05) +	"	"	(0.05)	...	5	4
Iso-butyl gallate (0.05) +	tartaric acid	(0.05)		...	10	9
Ethyl gallate (0.05) +	"	"	(0.05)	...	6.5	5.5
Iso-amyl gallate (0.05) +	"	"	(0.05)	...	5	4
Propyl gallate (0.05) +	"	"	(0.05)	...	6	5
Iso-butyl gallate (0.05) +	oxalic acid	(0.05)		...	9	8
Ethyl gallate (0.05) +	"	"	(0.05)	...	7	6
Iso-amyl gallate (0.05) +	"	"	(0.05)	...	6	5
Propyl gallate (0.05) +	"	"	(0.05)	...	7.5	6.5
Iso-butyl gallate (0.05) +	malonic acid	(0.05)		...	8.5	7.5
Ethyl gallate (0.05) +	"	"	(0.05)	...	7	6
Iso-amyl gallate (0.05) +	"	"	(0.05)	...	5	4
Propyl gallate (0.05) +	"	"	(0.05)	...	6	5
Iso-butyl gallate (0.05) +	succinic acid	(0.05)		...	8	7
Ethyl gallate (0.05) +	"	"	(0.05)	...	6	5
Iso-butyl gallate (0.05) +	hydroquinone	(0.05)		..	7.5	6.5
Ethyl gallate (0.05) +	"	(0.05)		...	6	5
Iso-butyl gallate (0.05) +	maleic acid	(0.05)		...	4	3
Ethyl gallate (0.05) +	"	"	(0.05)	...	3	2
Iso-amyl gallate (0.05) +	"	"	(0.05)	...	3	2
Iso-butyl gallate (0.05) +	sodium dihydrogen phos- phate (0.05)	7	6

The synergetic effect of certain mixtures of inhibitors is reported by a number of workers in the literature, e.g. orcinol and phosphoric acid on lard (Olcott and Mattill, *loc. cit.*), hydroquinone and lecithin on halibut-liver oil (Holmes, Corbet and Hartzler, 1936), Kamala dye and oleic acid on ghee (Govindarajan and Banerjee, 1939). So it was thought desirable to study the effect of some combinations of the best phenolic and acid-type inhibitors on shark-liver oil. The results obtained are summarized in Table II and some of the important curves are shown in Graph 2:—

GRAPH 2.

Synergetic effect at 75°C.



- | | | | |
|------|---------------------------|---|--------------------------------------|
| (1) | Control oil. | | |
| (2) | Iso-butyl gallate (0.05%) | + | maleic acid (0.05%). |
| (3) | " | + | sodium dihydrogen phosphate (0.05%). |
| (4) | " | + | hydroquinone (0.05%). |
| (5) | " | + | succinic acid (0.05%). |
| (6) | " | + | malonic acid (0.05%). |
| (7) | " | + | oxalic acid (0.05%). |
| (8) | " | + | tartaric acid (0.05%). |
| (9) | " | + | citric acid (0.05%). |
| (10) | " | + | ortho-phosphoric acid (0.05%). |

To confirm the results obtained by the oxygen-absorption tests and to study the vitamin A destruction, mildly accelerated storage tests with the more promising anti-oxidants were carried out by exposing thin layers of the oil to atmospheric oxidation

in flat-bottomed specimen tubes at 40°C. (Dattatreya Rao, 1945). After regular intervals, three tubes of each set were taken out, the oils contained in them were mixed together and immediately vitamin A was estimated by the Carr-Price reaction using the Pulfrich photometer with filter S. 61 (Dattatreya Rao, 1944). The induction periods were read off from the curves drawn with time in days as abscissæ and loss in vitamin A (per cent) as ordinates. The results are summarized in Table III and the curves are shown in Graphs 3 and 4 :—

TABLE III.
*Stabilization of vitamin A by single or combinations of anti-oxidants
at 40°C.*

Anti-oxidants added and concentration (per cent).					Induction period in days.*	Protection factor.
Control	1	...
Ortho-phosphoric acid (0.02)	2	1
Gallic acid (0.02)	2.5	1.5
Methyl gallate (0.02)	3.5	2.5
Hydroquinone (0.02)	4	3
Ethyl gallate (0.02)	7	6
Propyl gallate (0.02)	5	4
Iso-amyl gallate (0.02)	5	4
Iso-butyl gallate (0.02)	9	8
„ „ (0.02) + maleic acid (0.01)	6.5	5.5
„ phate (0.01) (0.02) + sodium dihydrogen phos- phate (0.01)	11	10
Iso-butyl gallate (0.02) + hydroquinone (0.01)	13	12
„ „ (0.02) + succinic acid (0.01)	13	12
„ „ (0.02) + malonic acid (0.01)	14	13

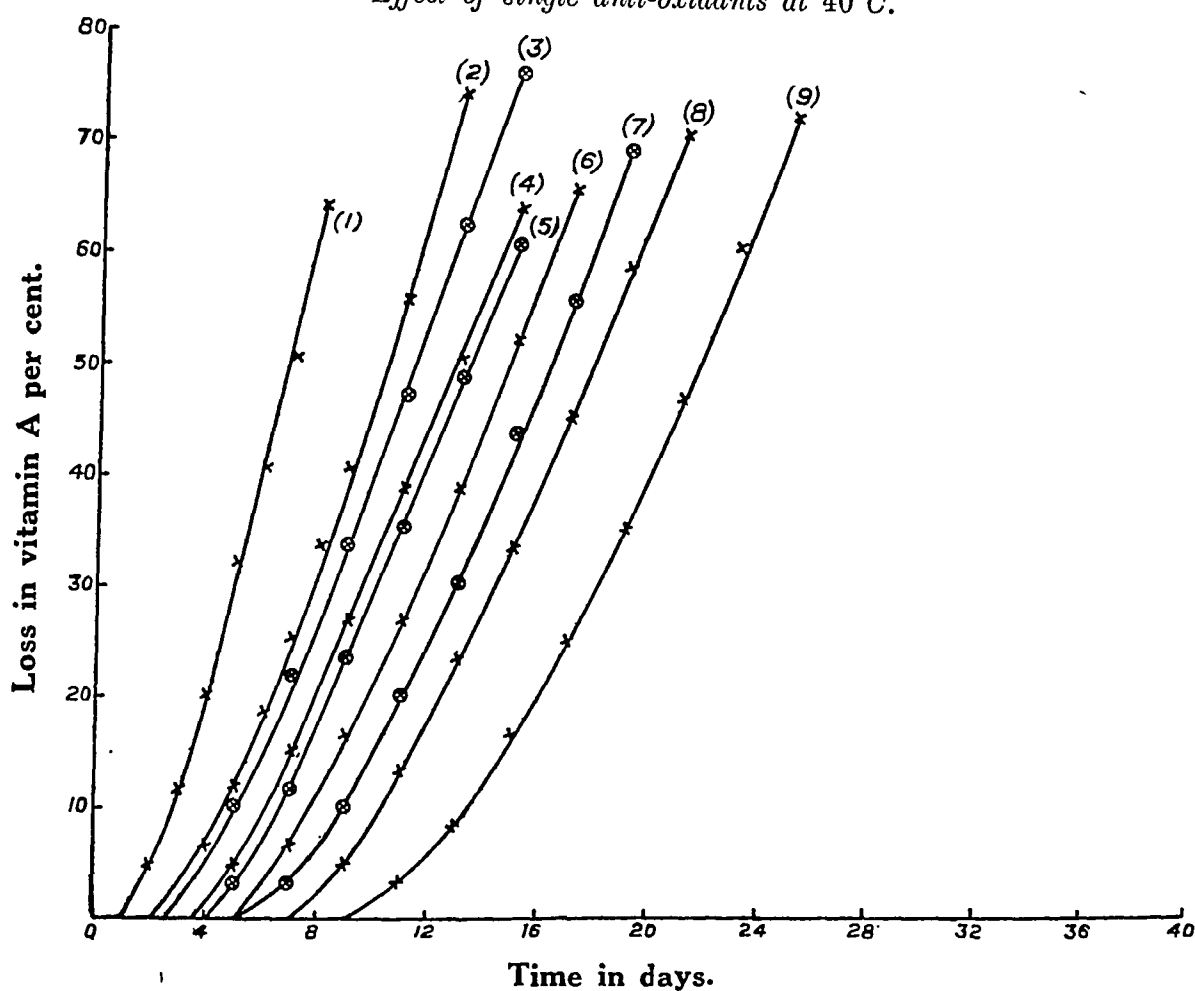
* Here 'induction period' refers to the time in days during which the destruction of vitamin A does not begin.

Anti-oxidants added and concentration (per cent).	Induction period in days.*	Protection factor.
Iso-butyl gallate (0.02) + oxalic acid (0.01)	16	15
" " (0.02) + tartaric acid (0.01)	17	16
" " (0.02) + citric acid (0.01)	17	16
" " (0.02) + ortho-phosphoric acid (0.01)	21	20

* Here 'induction period' refers to the time in days during which the destruction of vitamin A does not begin.

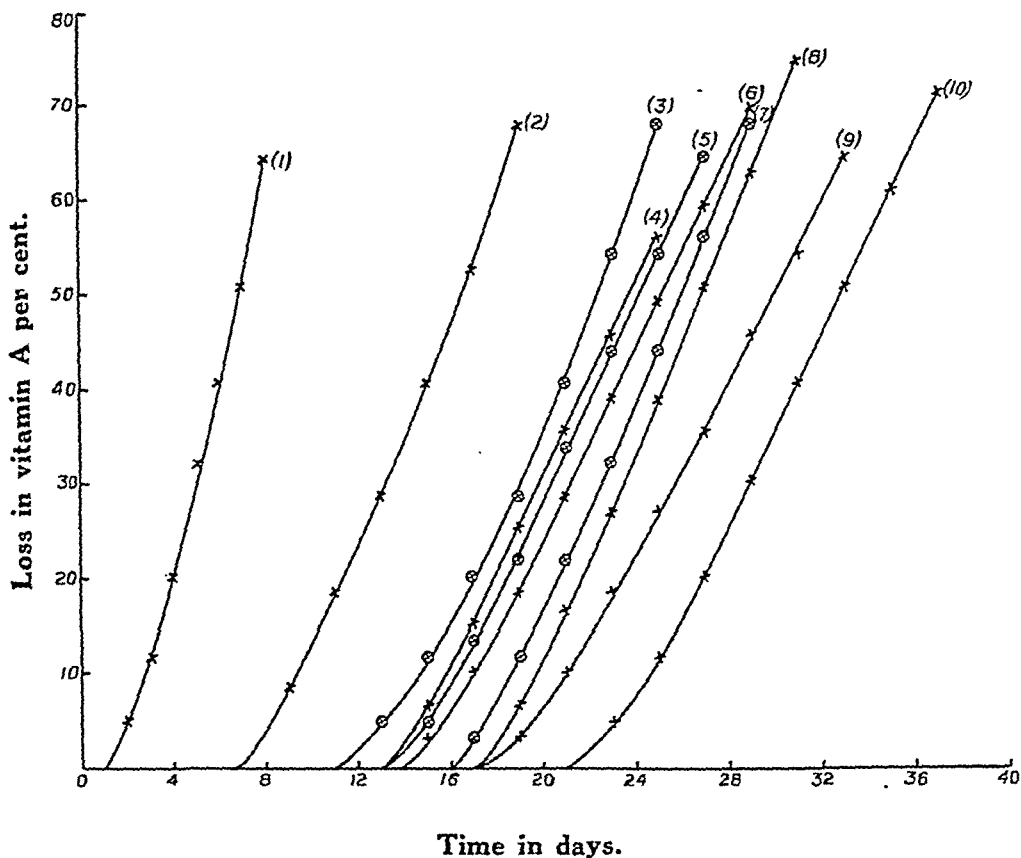
GRAPH 3.

Effect of single anti-oxidants at 40°C.



- (1) Control oil.
- (2) Ortho-phosphoric acid (0.02%).
- (3) Gallic acid (0.02%).
- (4) Methyl gallate (0.02%).
- (5) Hydroquinone (0.02%).
- (6) Propyl gallate (0.02%).
- (7) Iso-amyl gallate (0.02%).
- (8) Ethyl gallate (0.02%).
- (9) Iso-butyl gallate (0.02%).

GRAPH 4.

Synergetic effect at 40°C.

- | | | |
|------|---------------------------|--|
| (1) | Control oil. | |
| (2) | Iso-butyl gallate (0.02%) | + maleic acid (0.01%). |
| (3) | " " | + sodium dihydrogen phosphate (0.01%). |
| (4) | " " | + hydroquinone (0.01%). |
| (5) | " " | + succinic acid (0.01%). |
| (6) | " " | + malonic acid (0.01%). |
| (7) | " " | + oxalic acid (0.01%). |
| (8) | " " | + tartaric acid (0.01%). |
| (9) | " " | + citric acid (0.01%). |
| (10) | " " | + ortho-phosphoric acid (0.01%). |

DISCUSSION AND SUMMARY.

While carrying out the storage tests, it was observed that there was no appreciable change of odour and taste during the induction periods of the oil samples; soon after, the oils developed a characteristic taste and sharp odour indicating that the onset of rancidity usually coincides with or follows shortly after the end of the induction period, and also the destruction of vitamin A generally proceeds as the oil develops rancidity.

Accelerated oxidation tests can give clear comparative estimates of the stability of the oils under actual storage conditions (Orson Bird, 1942). Recently, Lea (1944) has shown that the rapid 'sorting' tests at 100°C. and the storage tests at 37°C. agree well on the whole, in placing the various anti-oxidants in order of their efficiencies. In our experiments also, there is fairly good agreement between the relative potencies of the inhibitors, as observed at the two temperatures 75°C. and 40°C.

Most of the acid-type inhibitors and the amino-acids practically fail to protect the oil, with the exception of ortho-phosphoric acid. Among the phenolic inhibitors, hydroquinone protects vitamin A for a considerable period. The esters of gallic acid have proved more effective, the order of efficiency of the gallates and gallic acid being iso-butyl gallate, ethyl gallate, iso-amyl gallate, propyl gallate, methyl gallate, and gallic acid.

The synergetic effect of the gallates and the acid-type inhibitors is obvious (Table III and Graph 4). The maximum inhibiting action is obtained with a mixture of iso-butyl gallate and ortho-phosphoric acid; but the oil loses its original colour and becomes darker and a black deposit is formed at the bottom of the specimen tubes. This is a drawback in actual practice. By using the salt, sodium dihydrogen phosphate, the difficulty of discoloration does not arise, but the inhibiting action is much retarded.

The combination of iso-butyl gallate and citric or tartaric acid is next best in merit. This combination does not discolour the oil, nor impart any foreign flavour to it. The occurrence of citric and tartaric acids in our everyday foodstuffs is well known and hence no objection can be raised to their use in shark-liver oil. The gallates also are usually considered non-toxic. The non-toxicity of propyl gallate has been indicated by Boehm and Williams (*loc. cit.*). Gunn, and Macdonald (quoted by Hilditch, *loc. cit.*), have reported the non-toxicity of ethyl gallate. There is, however, no such record of the non-toxicity of iso-butyl gallate in the literature; but for the very low concentration used, it is expected that the gallate will not be harmful. As suggested by Boehm and Williams (*loc. cit.*), under actual storage conditions, far lower concentrations of anti-oxidants may provide sufficient protection to the oil.

There is a slight drawback in using the gallates and the organic acids in shark-liver oil, viz. they are not perfectly soluble in the oil at room temperature. It has been observed, however, that after incorporating the mixtures of iso-butyl gallate (0.02 per cent) and citric or tartaric acid (0.01 per cent) in the oil, if it is kept for about 3 hours at 40°C., they completely dissolve in it and do not again settle out.

The preparation of the gallates is easy and inexpensive. The esterification of the homologues can be achieved by dissolving gallic acid in the corresponding hot alcohol, saturating the solution with dry hydrochloric acid gas and then refluxing for some time.

Hence, the combination of iso-butyl gallate and citric or tartaric acid appears to be the best anti-oxidant, so far examined, for shark-liver oil. There is, however, scope for further investigation for selecting better preservatives.

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VITAMIN A CONTENT OF THE LIVER OILS OF COMMON FRESH-WATER FISHES OF THE PUNJAB.

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INTRODUCTION.

THE vitamin A content of the liver oils of Indian fishes have been reported by a number of investigators from different parts of the country (Chakravorty, Mukerji and Guha, 1933; Ghosh, Chakravorty and Guha, 1933; Datta and Banerji, 1934; De, 1936; De, Majumdar and Sundararajan, 1938; and others). No work has been reported so far from the Punjab. The livers of common fresh-water fishes available in the Punjab have now been examined and data on the vitamin A content of their oils is reported in this paper.

The fishes were obtained from the fish markets in Lahore, and were generally caught from the river Ravi and the neighbouring streams. The fish examined weighed from 0.5 kg. to 4.0 kg. yielding a liver tissue 11.0 g. to 15.4 g. per kg. body-weight. When the livers were small, the oils were extracted from a number of livers of the same species and nearly of the same weight of the fish. The livers were ground with about 10 times their weight of anhydrous sodium sulphate and the dry powder so obtained was extracted in a Soxhlet apparatus for 4 to 5 hours with peroxide-free diethyl ether. The oils obtained were viscous brown liquids at winter room temperature of 16°C. to 18°C.

(a) *Colorimetric estimation.*—Colorimetric estimations were made according to the British Pharmacopœia (1932) method using a Lovibond tintometer, B. D. H. pattern. Dilutions of the oil taken were such as to give 4 to 6 blue units of the tintometer reading.

(b) *Spectrophotometric estimation.*—A Hilger E.4.302 Quartz Spectrograph was used with a photometer arrangement devised by the authors in the laboratory. A hydrogen-discharge tube used as the source of light was also made in the laboratory by the authors. $E_{1\text{ cm}}^{1\%}$ values of the whole oil were determined using absolute ethyl alcohol as the solvent. In view of the high potency of the oils it was not necessary to use the unsaponifiable matter of the oils.

The colorimetric estimations were carried out the same day as the liver oils were extracted, but the spectrographic estimations were undertaken 8 months after the tintometric estimations, when the spectrographic equipment was ready. During this interval the oils were stored in small stoppered-bottles at room temperature in winter and in a refrigerator during the summer months.

The results are recorded in the Table. To convert E value into I. U. of vitamin A, 1,600 has been used as the conversion factor in conformity with the practice of British workers. American workers, however, suggest the use of 2,000 as conversion factor (Coy, Sassaman and Black, 1941).

The data summarized in the Table show that the vitamin A content of the liver oils of the fresh-water fishes of the Punjab varies generally from 10,000 I. U. to 26,000 I. U. per g. of oil. A value of approximately 10,000 I. U. per g. of the liver oil was, however, obtained in case of only one species, namely Rôhu (*Labeo rohita*). Shingaree fish (*Mystus aor*) yielded liver oil with a vitamin A content as high as 26,400 I. U. per g. With the exception of Rôhu (*Labeo rohita*) the average vitamin A content of most other species

TABLE

Name of the fish.	Weight of fish, kg.	Weight of liver, g.	Average weight of oil per 100 g. liver.	Colour of oil.	Carr-Price value.	E 1 % E 1 cm.	L. U. per g. of oil.	Carr-Price value E 1 % value. E 1 cm.
Halibut (<i>Hippoglossus vulgaris</i>)	...	Halibut oil manufactured by Crooke's Laboratories, England, for comparison.						
(i)	...	2.13	21.3	2.5	1,685	32.1	51,360	52.5
(ii)	...	0.9	7.1		1,721	31.9	51,040	54.0
	0.9	7.1						
	0.75	10.5			951	10.5	16,800	90.5
	1.1	10.8						
Shingaree (<i>Mystus aor</i>)—				Yellowish brown.	949	14.78	23,650	64.2
(iii)	...	1.12	6.4		1,177	16.5	26,400	71.3
	0.7	4.0						
	0.7	3.2						
(iv)	...	0.5—2.0 (5 fish)	48 (5 livers)	6.40	1,237	6.27	10,030	197.0
(v)	...	2.0	14.5 (2 livers)	5.40	436	9.79	15,950	43.5
	1.3							
Mullee (<i>Wallagonia altu</i>)—								
(i)	...	1.6	19.5	2.53	1,040	12.97	20,750	80.0
	1.6	17.5						
(ii)	...	2.0	32.5	2.69	1,140	15.28	24,400	74.0

(iii)	1.3	10.4	4.1	Dark brown	507
(iv)	149.0 (several livers)	3.1	Yellowish brown.	854	13.45	21,580 62.5
(v)	0.9	14	}	"	800	12.06	19,300 66.3
		1.12	24						
		1.12	21						
		0.9	14						
		0.9	12						
		0.8	10						
(vi)	54	4.9	Yellowish brown.	934	8.87	14,192 105.0
phita)—									
(i)	0.6 each (5 fish)	28 (5 livers)	7.1	Dark brown	418	7.19	11,500 58.0
(ii)	0.5—1.0 (5 fish)	31.4 (5 livers)	8.54	"	413	6.66	10,650 63.5
tita)—									
(i)	0.5 each (5 fish)	16.2 (5 livers)	3.83	Yellowish brown.	614	13.31	21,300 47.0
(ii)	0.8	10	}	"	876	10.82	17,312 81.0
		0.8	11						
Joining liver of Mullet									
(vii)	Colourless	Trace

was between 20,000 I. U. and 26,000 I. U. per g. of the liver oil. This showed that these liver oils were among the richest sources of vitamin A. The yields of the oil from the livers were generally between 2.5 and 4.1 per cent though in some cases higher yields up to 8.54 per cent were recorded. These yields are very low as compared with shark-liver oil which gives yields in the neighbourhood of 20 per cent. It will be noticed that in Rôhu where the yield of oil was high, the vitamin A content of the oil was low.

Somewhat lower values were reported by Ghosh, Chakravorty and Guha (*loc. cit.*) for the livers of fresh-water fishes in Bengal. Seshan (1940) obtained a value of 20,000 I. U. per g. of the liver oil in case of only one species, whereas the average value for the 18 samples examined by him was above 18,130 I. U. per g. The liver oils of the common fishes of the Punjab are therefore among the richer liver oils found in India.

The spawning season of the fish examined is from May to September. The samples of oils were extracted and examined during the months of January and February.

No correlation has been found between the size or the age of the fish and the vitamin A content of the liver oil obtained from them.

The ratio between C. P. value and $E_{1\text{ cm}}^{1\%}$ of most of the samples examined was found to be between 58 and 80, the mean value being about 65, which is higher than the reported value of 50 to 55 for fish-liver oils in general. Although a value as low as 43.5 was also obtained, most of the values are yet higher than 50, and in one case as high a value as 197 was obtained. The discrepancy can be accounted for by the fact that whole oil was used for all these estimations. Better concordance in results may be obtained when the non-saponifiable fraction of the oil is used. The oils did not yield a perfectly clear solution in absolute alcohol. It is also possible that storage of the oil for eight months may have brought about some change in the oil.

The fishes examined were those commonly consumed in the Punjab. When these are caught, their livers and internal viscera are removed on the spot and discarded. The large amount of the livers which are thrown away as waste could be utilized for the preparation of the medicinal fish-liver oils. This is of practical importance particularly as Iyengar and Mukerji (1939) have reported that 90 per cent of the fish-liver oils and vitamin A concentrates sold on the Indian market are either very deficient in vitamin A or below the B. P. standard for cod-liver oil.

SUMMARY.

The liver oils of some common species of fish found in the streams and rivers of the Punjab have been examined for their vitamin A content both by the colorimetric and spectrographic method of assay. They have been found to be rich sources of vitamin A. Values as high as 20,000 I. U. and 26,000 I. U. per g. have been recorded. The livers which are now allowed to go waste could yield appreciable quantities of medicinal fish-liver oils.

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THE INFLUENCE OF BUTTER FAT IN THE ABSENCE OR PRESENCE OF CASEIN ON GROWTH IN YOUNG RATS ON A RICE DIET.

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SINCE October 1942 experiments with rats have been conducted in the Nutrition Department of the Women's Christian College (i) to demonstrate for educational purposes the inadequacy of diets composed mainly of rice, (ii) to demonstrate and test further the effects of supplements to rice diets, (iii) to compare South Indian cereals, and (iv) to explore modifications and improvements of rice diets that do not involve appreciable increase in cost. The work has benefited from the beginning from the long experience with rice diets of the Nutrition Research Laboratories, I.R.F.A., at Coonoor, and from the advice and generosity of Dr. W. R. Aykroyd.

In the course of class experiments planned to demonstrate the nutritive importance first of milk and then of some of its separate constituents with rice diets, the well-known effects of milk and of calcium lactate in accelerating growth in rats were clearly confirmed. The effect of casein, however, was more marked than reported by Aykroyd and Krishnan (1937) and although the diet was markedly deficient in total fat and contained a negligible quantity of animal fat, the addition of butter to the poor Madrasi diet not only did not aid growth but if anything adversely affected both the growth and the general condition of the animals. The same results were observed with three vegetable oils commonly used in South India, coco-nut oil, gingelly oil and ground-nut oil, as well as with shark-liver oil (unpublished data).

The following is the report of an experiment planned to test these preliminary observations, viz. the effects of these three ingredients of milk: casein, calcium and butter, singly as well as combined.

GENERAL TECHNIQUE.

The rats used are Wistar stock, and are bred in the laboratory and fed on an abundant and well-balanced diet. The litters are kept on this diet in the cage with the mother until 4 weeks' age. After two or three days of separation from the mother, during which they are still on stock diet, they are used for experiments with rice

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† The author responsible for the statistical study.

diet. The basal rice diet used in the Women's Christian College is of the following composition :—

‘ The poor rice diet ’

	Per cent.
Parboiled milled rice	89.7
Dhal (<i>Cajanus indicus</i>)	3.0
Brinjal (<i>Solanum melongena</i>)	4.3
Green plantain	2.1
Gingelly oil (<i>Sesamum indicum</i>)	0.43
Mutton	0.26
Coco-nut	0.21

The diet contains approximately, per 100 g. uncooked foods, protein 6.5 g., fat 0.9 g., carbohydrates 74 g.; calcium 0.014 g., phosphorus 0.145 g., iron 2.32 mg.; total calories 329*.

This diet is in the main similar to the ‘poor Madrasi’ or ‘poor rice’ diet used in Coonoor with the important difference that parboiled rice is used instead of the raw rice of the Coonoor diets and that after the foods are weighed out in the proportions stated the rice and the dhal are cooked. The rice is *not washed* and *only as much water is used in cooking as can be absorbed*. In the process of cooking rice absorbs approximately $3\frac{1}{2}$ times its own weight of water. The cooked rice and cooked dhal are thoroughly mixed with the chopped raw ingredients and the oil, and all put once through a grinder.

It has been shown that most food factors present in rice are little affected by the degrees of heat used in cooking and that the considerable losses of minerals and vitamin B₁ and nicotinic acid during washing and cooking are recoverable in the washing and cooking water (Ranganathan, Sundararajan and Swaminathan, 1937; Aykroyd, Krishnan, Passmore and Sundararajan, 1940; Swaminathan, 1941, 1942). In this diet the rice is not washed and no cooking water is discarded so it may be considered that losses due to cooking are slight if not negligible.

The diet is given *ad lib.* but daily consumption of food is measured.

PLAN OF EXPERIMENT.

In order to test the individual and combined effects of casein, calcium lactate and butter on the growth in young rats fed on poor rice diet, an experiment was designed which conformed to a factorial experiment for $2 \times 2 \times 2$ levels (Fisher *et al.*, 1941, 1942, 1943). By this type of design it is possible to determine the effects of several factors on a fairly limited number of animals and yet maintain a high degree of precision.

Twenty-four young rats were distributed in eight cages, three in each cage. The three supplements were tried out in eight different ways, that is in all possible combinations. Table I gives the amount of supplements supplied, corresponding approximately to the quantities of protein, calcium and fat in 12.5 grammes of milk. One group of rats received no supplements, three groups received single supplements, three combinations of two and one group received all three supplements. By this arrangement it is possible to estimate the effect of a single supplement, e.g. butter, on the basis of 12 rats receiving butter and 12 rats not receiving butter, the two groups being treated alike except for the butter (Table II). Similarly, the rats can also be divided into two groups according to whether they have received casein or no casein, or calcium lactate or no calcium lactate. As will be shown later the interactions between the three supplements, either between pairs or between all three together, can also be ascertained by comparison between two groups of twelve rats each.

* Calculated from tables in *Health Bulletin* No. 23, Govt. of India Press, New Delhi, 1941.

TABLE I.

Results of growth experiments on rats fed on poor rice diet supplemented with casein, calcium lactate and butter.

Cage.	Supplements to the basal rice diet.	Sex.	Initial weight, g.	Average weekly gain, g.	Mean.	Relative growth rate, per cent per week.	Mean.
1.	Nil	...	$\left\{ \begin{array}{l} \text{F} \\ \text{F} \\ \text{M} \end{array} \right.$	$\left\{ \begin{array}{l} 41.5 \\ 41.0 \\ 30.0 \end{array} \right.$	$\left\{ \begin{array}{l} 0.39 \\ 1.16 \\ 0.16 \end{array} \right.$	$\left\{ \begin{array}{l} 1.52 \\ 2.64 \\ 1.30 \end{array} \right.$	1.820
2.	Casein, 0.4 g.	...	$\left\{ \begin{array}{l} \text{F} \\ \text{M} \\ \text{M} \end{array} \right.$	$\left\{ \begin{array}{l} 35.0 \\ 30.0 \\ 40.0 \end{array} \right.$	$\left\{ \begin{array}{l} 2.71 \\ 3.44 \\ 2.61 \end{array} \right.$	$\left\{ \begin{array}{l} 6.31 \\ 7.33 \\ 5.62 \end{array} \right.$	6.420
3.	Calcium lactate, 0.115 g.	...	$\left\{ \begin{array}{l} \text{F} \\ \text{F} \\ \text{M} \end{array} \right.$	$\left\{ \begin{array}{l} 37.0 \\ 31.5 \\ 42.0 \end{array} \right.$	$\left\{ \begin{array}{l} 1.72 \\ 3.67 \\ 2.61 \end{array} \right.$	$\left\{ \begin{array}{l} 4.60 \\ 8.69 \\ 5.19 \end{array} \right.$	6.160
4.	Butter, 0.5 g.	...	$\left\{ \begin{array}{l} \text{F} \\ \text{M} \\ \text{M} \end{array} \right.$	$\left\{ \begin{array}{l} 33.0 \\ 46.5 \\ 30.5 \end{array} \right.$	$\left\{ \begin{array}{l} -0.17 \\ -0.22 \\ 0.17 \end{array} \right.$	$\left\{ \begin{array}{l} 0.38 \\ -0.24 \\ 0.71 \end{array} \right.$	0.283
5.	Casein, 0.4 g. and calcium lactate, 0.115 g.	...	$\left\{ \begin{array}{l} \text{F} \\ \text{M} \\ \text{M} \end{array} \right.$	$\left\{ \begin{array}{l} 35.0 \\ 30.5 \\ 42.0 \end{array} \right.$	$\left\{ \begin{array}{l} 4.83 \\ 6.94 \\ 6.06 \end{array} \right.$	$\left\{ \begin{array}{l} 9.25 \\ 11.97 \\ 9.39 \end{array} \right.$	10.203
6.	Butter, 0.5 g. and casein, 0.4 g.	$\left\{ \begin{array}{l} \text{F} \\ \text{M} \\ \text{M} \end{array} \right.$	$\left\{ \begin{array}{l} 43.5 \\ 32.5 \\ 34.0 \end{array} \right.$	$\left\{ \begin{array}{l} 3.89 \\ 3.17 \\ 2.67 \end{array} \right.$	$\left\{ \begin{array}{l} 7.25 \\ 7.63 \\ 6.47 \end{array} \right.$	7.117
7.	Butter, 0.5 g. and calcium lactate, 0.115 g.	...	$\left\{ \begin{array}{l} \text{F} \\ \text{F} \\ \text{M} \end{array} \right.$	$\left\{ \begin{array}{l} 32.5 \\ 32.5 \\ 42.0 \end{array} \right.$	$\left\{ \begin{array}{l} 1.61 \\ 0.83 \\ 1.50 \end{array} \right.$	$\left\{ \begin{array}{l} 5.10 \\ 3.32 \\ 3.38 \end{array} \right.$	3.933
8.	Butter, 0.5 g., casein, 0.4 g. and calcium lactate, 0.115 g.	...	$\left\{ \begin{array}{l} \text{F} \\ \text{F} \\ \text{M} \end{array} \right.$	$\left\{ \begin{array}{l} 31.0 \\ 35.0 \\ 43.5 \end{array} \right.$	$\left\{ \begin{array}{l} 5.33 \\ 6.22 \\ 9.61 \end{array} \right.$	$\left\{ \begin{array}{l} 11.04 \\ 11.62 \\ 12.24 \end{array} \right.$	11.633

The rats used in this experiment were from four different stock litters. Not enough rats being available to keep a balance of sexes and litters, they were distributed at random except to ensure that the total weights of the animals per cage were approximately uniform and that each cage contained at least one animal of each sex.

The experiment lasted nine weeks.

EFFECTS OF SUPPLEMENTS ON GENERAL CONDITION OF ANIMALS.

On the poor rice diet alone the hair of the animals became loose and dry, and fell out or was chewed off by cage mates until neck and back were almost denuded. The gait and posture were affected, an effect commonly described by the workers in the laboratory as 'running on hind toes'. These are common characteristics of rats fed on this diet. The rats receiving casein appeared normal except for somewhat thinned coats. The general condition of the rats with butter was worse than that of those receiving no supplement at all: they were almost completely denuded of hair on head, neck and back; the hind legs became almost erect when standing, and the animals could rest only with the legs spread wide apart; the tails became scaly and ringed and were broken off in successive stages. Of the rats fed on single supplements the coats of those receiving calcium lactate were best.

On calcium lactate *plus* butter the coats were markedly thin; on casein and calcium lactate the coats were excellent; on casein and butter they were thinned in the first two weeks but recovered and were in good condition before the end of the experiment. The rats receiving all three supplements had excellent coats. No rats except those on the poor rice diet alone and those on butter supplement alone showed deformity of the hind legs, and only in the butter cage were the tails affected.

METHOD OF STATISTICAL ANALYSIS.

The statistical significance of the experiment was based upon a study of the logarithmic growth curves in preference to the absolute growth curves, and the *proportionate* weight gains, expressed as the average relative growth rates per week, were submitted to statistical analysis.

(a) *Relative growth rates.*—The advantage of analysing relative growth rates instead of the frequently used average absolute gains is evident if it is considered that two animals with the same absolute growth rates would have grown quite differently if they started from different initial weights. Two rats from a recent experiment may be taken as an illustration of this fact, the one weighing 47 g., the other 32 g. at the beginning of the experiment. Their weights after nine weeks were 101.5 g. and 89 g. respectively, giving average weekly gains of 6.06 g. and 6.33 g. The difference is apparently quite unimportant. Against this a marked difference was found when the proportionate gains were considered. While the weight of the larger rat was doubled, that of the smaller animal was nearly trebled—unquestionably a much better performance. The true difference was borne out by the average relative growth rates which were 8.40 and 12.19 per cent respectively.

The average absolute as well as the average relative growth rates for each of the animals are given in Table I. The former, expressed in grammes, was calculated as the simple arithmetic mean of the weekly gains, whereas the latter, the relative rate, was found by determining the regression coefficient of the straight line fitted to the logarithmic growth curve (to base *e*). This coefficient when based upon natural logarithms indicates the rate of increase per unit of time as well as per unit of weight already attained; multiplied by 100 it expresses the percentage rate of increase per week (Fisher, 1941). A discussion of the shape of the logarithmic growth curves is given in the *Appendix*.

(b) *Analysis of variance.*—In Table II the relative growth rates per week of all the rats are arranged according to (i) different supplements, and (ii) initial weights, the first column showing the growth rates of the smallest animals, the third those of the largest. As already pointed out the three supplements were tried out in all possible combinations, that is in eight ways. The effect of a single supplement, e.g. butter, is estimated by comparing the mean relative growth rate of all twelve animals receiving butter with that of the remaining twelve which did not get it. As the total of the relative growth rates of the first 4 groups of rats in Table II which received no butter is 73.81 against 68.90 of the last 4 groups, the difference is -4.91; this divided by 12 gives the effect of butter as -0.41 per cent (as shown in Table IV). Each of the three interactions between two supplements, e.g. casein \times butter, and the one triple-action (casein \times butter \times calcium lactate) is similarly analysed on the basis of all 24 animals.

Taking the former as an example, the two groups which received casein and butter simultaneously (one with calcium lactate in addition, the other without calcium lactate) *plus* the two groups receiving no casein or butter may be compared with the remaining four groups which got either casein or butter but not simultaneously; the mean relative growth rates per rat are 6.68 and 5.21 per cent respectively. The difference is +1.47 (as in Table IV), and it expresses the interaction effect between butter and casein; being positive it indicates that the growth is accelerated 1.47 beyond what would be expected by the mere summing of the single effects of butter and casein.

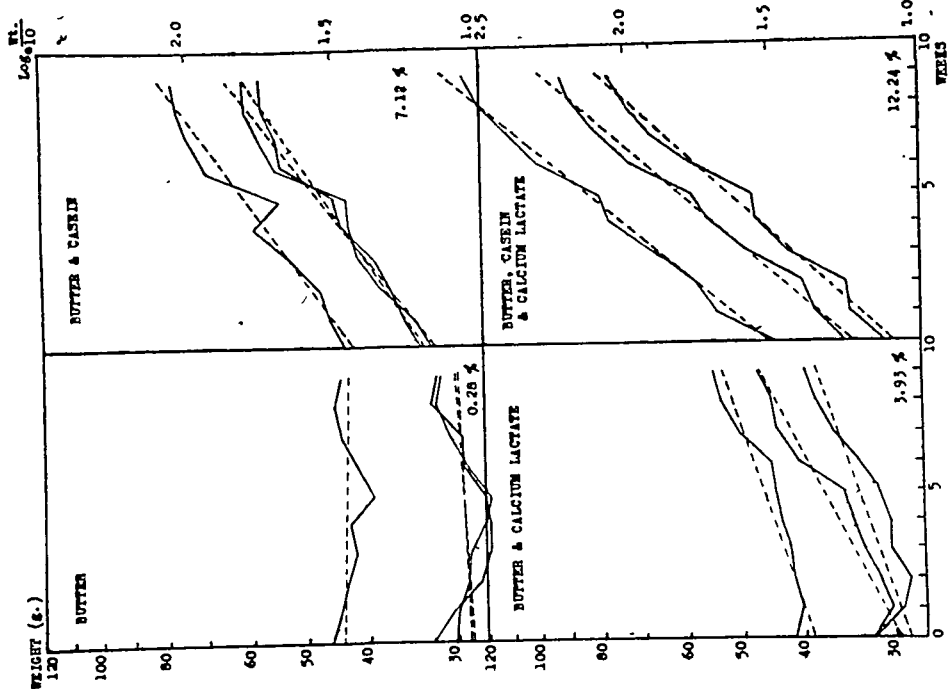


Fig. 2. Logarithmic growth curves of rats fed on a basal rice diet with a supplement of butter with or without casein and calcium lactate. The straight lines indicate the regression lines fitted to the curves. The percentage figures are the means of the average weekly relative growth rates.

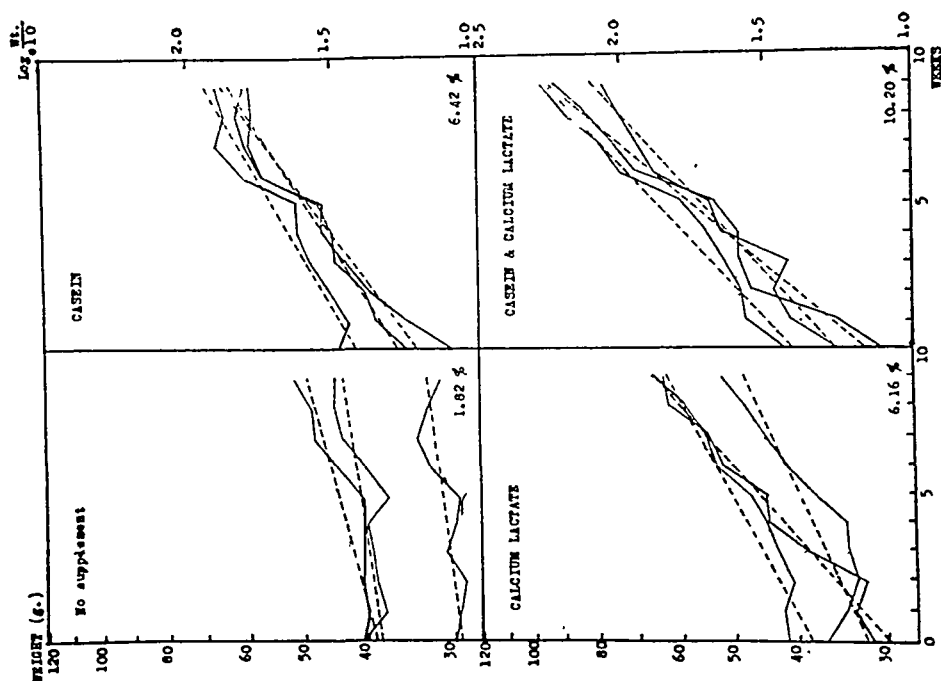


Fig. 1. Logarithmic growth curves of rats fed on a basal rice diet with supplements of casein and calcium lactate. The straight lines indicate the regression lines fitted to the curves. The percentage figures are the means of the average weekly relative growth rates.

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Taking the former as an example, the two groups which received casein and butter simultaneously (one with calcium lactate in addition, the other without calcium lactate) *plus* the two groups receiving no casein or butter may be compared with the remaining four groups which got either casein or butter but not simultaneously; the mean relative growth rates per rat are 6.68 and 5.21 per cent respectively. The difference is +1.47 (as in Table IV), and it expresses the interaction effect between butter and casein; being positive it indicates that the growth is accelerated 1.47 beyond what would be expected by the mere summing of the single effects of butter and casein.

Before discussing the significance of the supplementary effects, attention may be drawn to the observation that the growth rates were affected by the difference in initial weights, the smaller rats growing faster than the larger ones. This observation, though statistically not quite of significance value, is in keeping with common experience from nutrition experiments with other species of animals. Although it has no direct bearing upon the chief purpose of the present investigation, the importance of the observation lies in the possibility of increasing the efficiency of the experiment by separating from the residual sum of squares (Table III) the part contributed by the variation caused by different initial weights. By reducing the residual sum of squares on which is based the estimate of the experimental error, the latter becomes smaller and, therefore, the precision of the experiment higher. In this connection it may be added that an estimate of the experimental error on basis of the *absolute* growth rates showed it to be about twice as large as in the present analysis, corresponding to a precision about four times less.

TABLE IV.

Main effects of supplements expressed in terms of average relative growth rate per week.

Supplements.				Relative growth rate per cent rat weight.
<i>Single effects :</i>				
Casein	+ 5.78*
Calcium lactate	+ 4.07*
Butter	- 0.41
<i>Interactions :</i>				
Casein × calcium lactate	+ 0.08
Casein × butter	+ 1.47*
Calcium lactate × butter	+ 0.01
<i>Triple-effect :</i>				
Casein × calcium × butter	+ 0.36
<hr/>				
Estimate of standard error	± 0.4038
<i>Significance levels,</i>				
at 5 per cent	0.87
„ 1 „	1.20
„ 0.1 „	1.67

* Highly significant.

RESULTS OF THE STATISTICAL ANALYSIS OF GROWTH RATES.

Table IV gives the numerical values of the change in relative growth rates caused by the various supplements. The result of the experiment as found by the statistical analysis may be summarized as follows.

TABLE II.

Relative growth rates (per cent per week) resulting from feeding rats on poor rice diet with supplements of casein, calcium lactate and butter, singly and combined.

Supplements.					(i)†	(ii)	(iii)	Total.	Mean.
Nil	1.30*	2.64	1.52	5.46	1.82
Casein	7.33*	6.31	5.62	19.26	6.42
Calcium lactate	8.69	4.60	5.19*	18.48	6.16
Casein + calcium lactate	11.97	9.25*	9.39*	30.61	10.20
Butter	0.71*	0.38	-0.24*	0.85	0.28
„ + casein	7.63*	6.47*	7.25	21.35	7.12
„ + calcium lactate	5.10	3.32	3.38*	11.80	3.93
„ + casein + calcium lactate	11.04	11.62	12.24*	34.90	11.63
TOTAL					53.77	44.59	44.35	142.71	...
MEAN					6.72	5.57	5.54	...	5.946

* Male rats.

† In column (i) are entered rats with the smallest initial weights, in (iii) those with the largest initial weights.

The result of the analysis of variance is given in Table III:—

TABLE III.

Analysis of variance.

Items.					Sums of squares.	D.F.	M. Sq.	V.R.	Probability.
<i>Single effects :</i>									
Casein	201.4342	1	201.43	205.89	Less than 0.001
Calcium lactate	99.5115	1	99.51	101.71	„ 0.001
Butter	1.0045	1	1.00	1.03	0.40—0.30
<i>Interactions :</i>									
Casein × calcium lactate	0.0360	1	0.036
Casein × butter	13.0095	1	13.01	13.30	0.01—0.001
Calcium lactate × butter	0.0007	1	0.0007
<i>Triple-effect :</i>									
Casein × butter × calcium lactate	0.7597	1	0.76
Total treatment effect	315.7561	7
Between initial weights	7.2111	2	3.61	3.68	0.20—0.05
Error	13.6973	14	0.9784
TOTAL					336.6645	23

D.F. = Degrees of freedom.

M. Sq. = Mean square or variance.

V.R. = Variance ratio.

5. It is concluded that the poor rice diet is deficient in a factor or factors essential for the utilization of dietary fat and that this factor is supplied in technical casein.

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APPENDIX.

Discussion on the shape of the logarithmic growth curves.

The adoption of the average relative growth rates as basis of analysis presumes that the linear regressions by themselves were adequate expressions of the logarithmic growth curves, or in other words that the curves followed closely a straight line. This again means that the growth rate should be constant throughout the experiment and the experiment be terminated before the growth tends to cease. As may be seen from Figs. 1 and 2 the curves did generally follow a straight course but in some cases they were slightly parabolic. In order to assess the significance of the deviations from a straight line, polynomial regression lines of second order were fitted to each logarithmic curve according to Fisher and Yates' method, and the coefficients of the terms are entered in Table V. From the coefficients to the quadratic term (column c of Table V) it will be seen that two of the rats receiving casein as supplement showed slightly parabolic growth curves, the negative signs of the coefficients indicating an upwards convex curvature, whereas in the case of rats receiving poor rice diet only or supplements of butter, calcium lactate or both combined, the curves had a downwards convex curvature, the signs being positive, indicating that the rats to begin with lost weight but later picked up, gaining slowly. Except in the case of the rats receiving butter all the linear regression coefficients were positive and significant indicating a definite gain in weight, and in the case of the rats in cages 5, 6 and 8 these coefficients alone were sufficient to express the shape of the curves which, therefore, truly followed the course of straight lines. In the other cases where the parabolic regression coefficients also were statistically significant, their values were so small that with a single exception they may be considered quite unimportant compared with the values of the linear regression coefficients. Only in case of the butter rats in cage 4 was the linear regression insignificantly but the parabolic regression significantly different from zero, which indicates that the animals just maintained their weight despite a temporary loss.

Concluding, it seems justified in the analysis to ignore the deviations from the straight regression line and to rely only upon the first order regressions as expressed by the average relative growth rates.

TABLE V.

Coefficients of polynomial regression lines of the form $y = a + bx + cx^2$ fitted to the logarithmic growth curves (to base e) of rats fed on a poor rice diet with various supplements.

Cage.	Supplements.	Sex.	a	b	c
1.	Nil
		F	1.373	+ 0.01524*	+ 0.00452*
		F	1.432	+ 0.02635*	+ 0.00491*
		M	1.130	+ 0.01297*	+ 0.00069

* Coefficients significantly different from zero.

Casein and calcium lactate caused a marked and highly significant acceleration of the growth, whereas butter had a negative though not significant effect*. Of the three interactions of first order, two were small and insignificant, but the interaction between casein and butter was highly significant (+1.47 being more than three times the experimental error), indicating a stimulus of the growth beyond what would be expected from a mere summing of the single effects of the two ingredients. The interaction between all three supplements was not significant.

DISCUSSION.

The deficiencies of poor rice diets have been discussed by Aykroyd and Krishnan (*loc. cit.*) and Aykroyd, Krishnan, Passmore and Sundararajan (*loc. cit.*), who state that these diets are deficient in vitamin A, calcium and various factors in the vitamin B₂ complex. In their experiments with rats the addition of skimmed milk powder induced a striking improvement in growth rate but it was found that much of the effect of the milk supplement could be produced by calcium lactate or calcium phosphate alone, and since it was found that casein produced a smaller improvement of the growth rate the conclusion was drawn that protein deficiency is of relatively minor importance with rice diets. The addition of extra fat in the form of gingelly oil did not accelerate the growth, the observation being interpreted as 'suggesting that the rice-eater's low fat intake does not represent *per se* a serious problem'.

In the present experiment not only calcium lactate but also casein accelerated growth very markedly, the difference between the effects of casein and calcium being statistically insignificant. The most interesting findings, however, are first, that when butter was added to a rice diet, already deficient in fat, the rats were unable to utilize the fat, and not only did not grow but developed pathological symptoms more severe than, and in some respects different from those produced by the rice diet alone; secondly, that when casein was added to the butter, these pathological effects of the added fat did not appear and the beneficial effect of butter appeared as shown by acceleration of growth even greater than could be explained by the casein effect alone.

These results seem to indicate that the poor rice diet is deficient in a factor or factors essential for the normal utilization of dietary fat, and that this factor was supplied by the casein. In experiments with a basal diet so badly balanced and with such multiple deficiencies as the poor rice diet, any attempt at interpretation of results at this stage must be very tentative. That the explanation is probably not to be found in the nature of the fat supplied is indicated by the fact that gingelly oil which is rich in linoleic acid (Hoover, 1939) produced the same effect as butter (unpublished data referred to above). Of contaminants present in technical casein a calcium effect may be ruled out by the results of the present experiment with calcium lactate and butter; the possibility of the B₂-group vitamins cannot be ignored but the quantities present in the amount of casein supplied are so minute as to make this explanation unlikely. A possible factor is the amino-acid methionine supplied by the casein.

SUMMARY.

1. The purpose of the present investigation was to examine the supplementary effects of casein, calcium lactate and butter, singly or combined, on the growth of young rats fed on a basal rice diet similar to that common among the poor in South India.

2. Casein as well as calcium lactate was found to have a highly significant effect in promoting growth.

3. Butter affected both growth rate and general condition adversely.

4. Casein when added to butter counteracted the negative effect of butter and converted it into a positive effect. This interaction was highly significant. There was no significant interaction between calcium lactate and butter or between calcium lactate and casein.

* The growth on the poor rice diet alone was so slight that a negative effect great enough to be significant would have resulted in the death of the animals.

INVESTIGATIONS ON THE NUTRITIVE VALUE OF TAPIOCA (*MANIHOT UTILISSIMA*).

BY

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TAPIOCA is extensively cultivated in various tropical countries. It is usually called cassava or manioc, the term 'tapioca' being applied to starchy processed products obtained from the root. In India, however, the root itself is called tapioca. Introduced into Travancore during the last century, tapioca is now extensively cultivated in that State and in neighbouring areas. In 1943-44 production in Travancore increased by some 33 per cent and the area under this crop reached 0.5 million acres out of a gross cultivated area of approximately 2.2 million acres. Total production in this year was estimated as 750,000 tons. During the war period the area under tapioca in Cochin State has doubled, production being 30,000 tons in 1943-44, while in Malabar the area has also been doubled, reaching in that year 40,000 acres, with an estimated production of 120,000 tons.

Certain characteristics of tapioca may be mentioned. It is a high-yielding dry crop, growing abundantly even on infertile soil, and does not need much attention. It has, in fact, been called a 'lazy man's crop'. The average yield in India is about 2 tons per acre, but yields as high as 10 to 15 tons per acre have been reported in other countries. Its calorific value is relatively high, and the return of calories per unit area from tapioca is higher than the return from rice or other cereals.

The root is mainly starchy and very poor in protein, containing less than 1 per cent. Its fat content is negligible and it is a poor source of minerals and vitamins of the B group. The fresh root contains appreciable quantities of vitamin C, but this is destroyed on drying. The leaves are a very rich source of ascorbic acid (Raymond, Jojo and Nicodemus, 1941), and according to van Veen (1938) 'thiamin, carotene and lactoflavin are present in more than average amounts'. The fresh root (as also the leaves) contains cyanogenetic glucosides and cases of tapioca poisoning in human beings and animals have been reported. Drying, however, completely destroys the glucosides, and if the roots are cooked in a fresh state the poison is extracted by the cooking water. In practice, human poisoning is rare in tapioca-eating areas.

Tapioca, as has been pointed out, is a food of growing importance in South India and careful investigation of its nutritive properties is called for. Preliminary work on the subject has been carried out in these Laboratories (Aykroyd and Krishnan, 1939). In the present study the nitrogen complex of the root, the *in vitro* digestibility of tapioca starch and the availability of its carbohydrates have been studied.

EXPERIMENTAL.

1. *The nitrogenous fraction.*

Fresh tapioca roots were stored in a refrigerator and used as required. For extraction of the nitrogen, they were made into fine paste and extracted with different solvents as described below. Slices weighing 100 grammes were ground well with 200 ml. of the solvent, the mixture transferred to a flask and shaken for 15 minutes. The mash was then squeezed through a muslin cloth and the extraction repeated thrice with the residue. The combined extracts were left in the refrigerator overnight to allow the starch to settle, and the supernatant decanted off and centrifuged to remove suspended matter. It was made up to volume, and aliquots taken for the various estimations.

TABLE V—*concl'd.*

Cage.	Supplements.	Sex.	<i>a</i>	<i>b</i>	<i>c</i>
2.	Casein, 0.4 g. ...	F	1.583	+ 0.06314*	— 0.00235*
		M	1.594	+ 0.07329*	— 0.00481*
		M	1.667	+ 0.05623*	+ 0.00125
3.	Calcium lactate, 0.115 g. ...	F	1.304	+ 0.04595*	+ 0.00894*
		F	1.487	+ 0.08692*	+ 0.00098
		M	1.540	+ 0.05189*	+ 0.00591*
4.	Butter, 0.5 g. ...	F	0.996	+ 0.00381	+ 0.00970*
		M	1.442	— 0.00239	+ 0.00418
		M	0.995	+ 0.00712	+ 0.01034*
5.	Casein, 0.4 g., and calcium lactate, 0.115 g.	F	1.683	+ 0.09253*	— 0.00019
		M	1.726	+ 0.11974*	— 0.00186
		M	1.790	+ 0.09387*	+ 0.00432
6.	Butter, 0.5 g., and casein, 0.4 g.	F	1.749	+ 0.07246*	+ 0.00049
		M	1.524	+ 0.07636*	— 0.00053
		M	1.385	+ 0.06473*	— 0.00106
7.	Butter, 0.5 g., and calcium lactate, 0.115 g.	F	1.279	+ 0.05099*	+ 0.00420*
		F	1.136	+ 0.03325*	+ 0.00724*
		M	1.484	+ 0.03376*	+ 0.00466*
8.	Butter, 0.5 g., casein, 0.4 g., and calcium lactate, 0.115 g.	F	1.582	+ 0.11044*	+ 0.00170
		F	1.846	+ 0.11622*	— 0.00114
		M	2.094	+ 0.12242*	— 0.00284

* Coefficients significantly different from zero.

Attempts were then made to determine the quantities of the various fractions of proteins present in the extracts by using known precipitants for albumins, globulins, etc. (Table III).

TABLE III.

Types of proteins present in extracts.

(N expressed as percentage of the total N extracted.)

Extract.	10 per cent trichloroacetic acid.	Half saturation with $(\text{NH}_4)_2\text{SO}_4$.	Full saturation with $(\text{NH}_4)_2\text{SO}_4$.	Heat coagulation at pH 7.0.	Precipitated at pH 4.0.
Water ...	28.57	Turbidity	Turbidity	Turbidity	10.71
5 per cent NaCl ...	41.86	„	27.90	13.95	4.65
70 per cent alcohol ...	Turbidity
0.2 per cent NaOH ...	35.16	9.89	17.58	...	18.68

It is to be noted that the water extract yielded no precipitate on heating. It was, however, found that some coagulation occurred at higher concentrations. But the coagulated protein again dissolved on cooling. It was possible to isolate the coagulum by immediately centrifuging it off while hot and drying by washing with alcohol, acetone and ether. In the case of precipitation with ammonium sulphate, though proteins were not precipitated in any appreciable quantity in these experiments, a precipitate was obtained when more concentrated extracts were used. This behaviour seems somewhat strange, but it may be due to the peculiar nature of the proteins in tapioca. The 70 per cent alcohol extract yielded no precipitate with trichloroacetic acid. A little precipitate (the precipitated N amounting to 0.395 per cent of the total N extracted) was obtained when the extract was diluted with water. This suggests that the proteins of tapioca may have different properties from those of the typical albumins, globulins, etc. The material was then extracted successively with different solvents: Fifty grammes of the tapioca slices were crushed well and the paste successively extracted with 150 ml. of water, 5 per cent sodium hydroxide, the shaking period being in each case 15 minutes. The extraction with each solvent was carried out three times, after which, as the preliminary experiments showed, the part of the N extractable with that solvent was completely removed. The combined extracts were centrifuged to remove suspended particles, and aliquots were used for nitrogen determinations. The results are shown below:—

Experiment.	Water.	5 per cent NaCl.	70 per cent alcohol.	0.2 per cent NaOH.
I	57.18	4.13	0.68	42.75
II	54.41	3.44	0.688	43.44

(N expressed as percentage of total N)

On comparing these results with the results obtained when separate portions of the material were extracted with different solvents, it can be seen that about 56 per cent of the

TABLE I.

Extraction of nitrogen from tapioca.

(N expressed as percentage of the total N in the root.)

Solvent.		First extraction.	Second.	Third.	Fourth.	Total N extracted.	N left in the residue.	N recovery as percentage of total.
Water	...	61.21	17.34	2.74	0.31	81.60	16.78	98.38
5 per cent NaCl	...	63.73	17.68	3.00	0.39	84.8	19.25	104.05
70 per cent alcohol	...	43.18	8.44	3.38	0.75	55.75	45.86	101.61.
0.2 per cent NaOH	...	72.81	16.91	6.93	0.23	96.88	5.40	102.28

It is evident from Table I that a certain proportion of the nitrogen was not extracted by the different extractants. This was rather high in the case of extraction with 70 per cent alcohol. During the preliminary experiments, the same was found to be the case when acid (1 per cent HCl) was used for extraction. The amount of nitrogen left unextracted in the residue, which gave positive protein colour reactions, varied with different solvents. It is of interest to note in this connection the close analogy with the behaviour of nitrogen in potato (Neuberger and Sanger, 1942). Though a portion of this unextractable nitrogen was of protein nature, the presence of other nitrogenous substances is also probable.

The protein fraction in the extracts was determined by precipitating it by a number of reagents. Table II summarizes the results :—

TABLE II.

Protein fractions in extracts.

(N expressed as percentage of the total N extracted.)

Precipitant used.	Percentage of soluble N precipitated.	Precipitant used.	Percentage of soluble N precipitated.
Trichloroacetic acid (10 per cent).	28.57	Lead acetate (20 per cent).	32.50
Ethyl alcohol (50 per cent).	27.50	Copper hydroxide (Stutzer's reagent).	10.00
Saturated barium hydroxide solution.	21.25	Phosphotungstic acid (5 per cent).	26.60

Lead acetate precipitated more N than the other typical protein precipitants, probably because it formed insoluble complexes with molecular nitrogenous substances in the extract, like the amino-acids and the nitrogenous bases. Copper hydroxide behaved in a different way, precipitating the least amount of soluble N; but this is only to be expected since this reagent is known to precipitate only the true proteins.

1930). These results together with the amino-acid composition of other proteins are given below :—

TABLE IV.
Amino-acid composition of tapioca protein and other proteins.

Protein of	Arginine N.	Tyrosine N.	Tryptophane N.	Cystine N.	Author.
Cabbage ...	13·0	2·2	1·2	1·1	Kao, Adolph, Liu (1935).
Sweet potato ...	11·8	2·4	1·8	1·8	„ „ „ „
Egg-albumin ...	10·7	2·1	1·2	1·0	Calvery (1931).
Casein ...	3·8	6·5	2·2	0·3	Sherman (1942).
Rice ...	13·7	...	0·9	1·1	Rosedale (1939).
Tapioca ...	17·0	1·6	1·1	1·3

It is to be seen from Table IV that the proteins of tapioca contain fair amounts of the important amino-acids and compare favourably with casein, egg-albumin and proteins of cabbage and sweet potato. It is, therefore, likely that the defect of tapioca lies in the low total quantity of protein contained in it rather than in its quality.

Analysis of non-protein nitrogen.—Many foodstuffs contain considerable amounts of non-protein nitrogen (McCance and Shipp, 1933 ; Bhagvat and Sreenivasaya, 1935) part of which may exist as simple derivatives of proteins and amino-acids, not precipitated by most of the common protein precipitants. Very little is known of the rôle of the non-protein nitrogen of foodstuffs, in nitrogen metabolism. The evidence available is contradictory. Bhagvat and Sreenivasaya (*loc. cit.*) state that the non-protein nitrogen of pulses consists of easily digestible and assimilable peptides ; while Swaminathan (1938) found that the non-protein nitrogenous materials (not precipitated by copper hydroxide) are not fully digested and contribute to the low digestibility coefficient of the nitrogen of certain foodstuffs. Analysis of the non-protein nitrogen in the water extracted of tapioca gave the following results :—

TABLE V.
Non-protein nitrogen in tapioca.
(Values as percentage of the total N extracted.)

Amide N ...	1·46	<i>Precipitated by phosphotungstic acid.</i>	
Total N ...	21·8		
Amino N ...	16·03		
Non-amino N ...	3·19		
<i>Not precipitated by phosphotungstic acid.</i>			
Total N ...	59·42	Histidine N ...	4·52
Amino N ...	48·03	Lysine N ...	11·58
Non-amino N ...	10·14	Cystine N ...	2·02
		Arginine N ...	3·7
		Tyrosine N ...	1·092
		Tryptophane N ...	0·581

The above results show that the digestibility of the proteins of tapioca was not low compared to those of rice and that they are digested without much difficulty by individual proteases. They were as readily digested by tryptic enzymes as rice proteins, and more readily by pepsin. The tapioca proteins were digested to a greater extent by papain than those of potato.

3. Carbohydrates.

Finely powdered tapioca root was used. The whole root rather than separated starch was taken because in the body the digestion of the starch takes place in the presence of other nutrients. The digestive enzymes employed were taka diastase and pancreatic amylase. For the sake of comparison similar *in vitro* experiments were carried out with wheat, rice and potato.

FIG. 1.

FIG. 2.

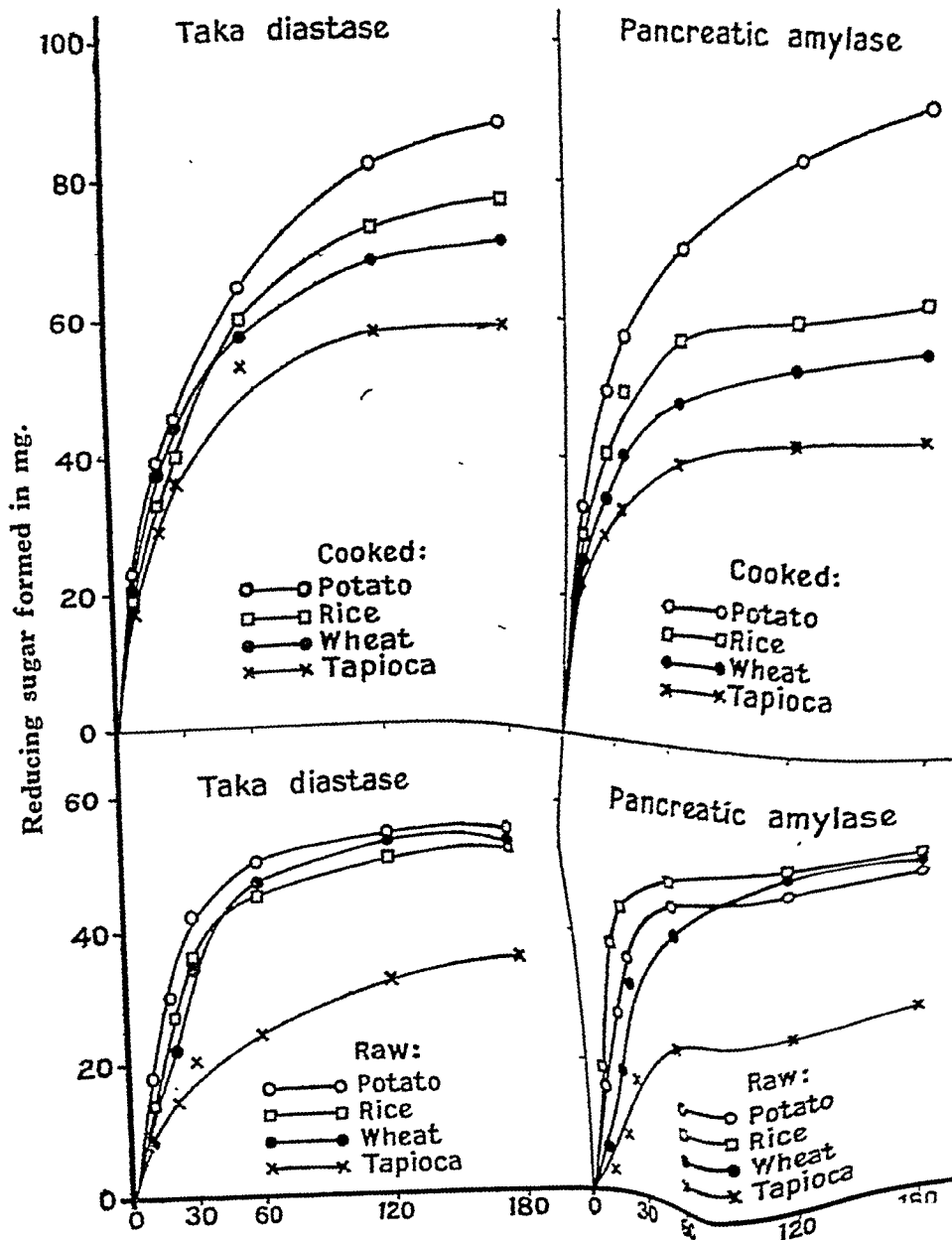


FIG. 3.

Time in minutes.

FIG. 4.

Digestibility of the starches in the raw state.—A weighed quantity (10 g.) of the finely powdered material was ground up thoroughly with water into a uniform paste and suspended in 120 ml. of phosphate buffer of pH 8.0 in experiments with taka diastase, and pH 6.9 in experiments with pancreatic amylase. In the latter case, 2 ml. of 0.2 N sodium chloride were also added to activate the enzyme. The experiments were carried out at a temperature of 40°C. When the contents of the flask attained this temperature, 10 ml. of a 2 per cent solution of the enzyme were added and the mixture stirred well. Aliquots of the reaction mixture were removed at intervals of 10, 20, 30, 60, 120 and 180 minutes and the reaction stopped by the addition of 2 ml. of normal hydrochloric acid. The reducing sugar formed was then determined by the hypiodite method of Willstatter, Waldschmidt-Leitz and Hesse (1923).

Digestibility of the starches after cooking.—The digestibility of the different starches was also determined on the cooked samples. The cooking was carried out for a period of 15 minutes in each case. The results are given in Tables VIII, IX and X and graphically shown in Figs. 1 and 2.

TABLE VIII.

Digestibility of various starches by taka diastase (in vitro).
(The reducing sugar formed is expressed as mg. of maltose.)

Time in minutes.	TAPIOCA.		RICE.		WHEAT.		POTATO.	
	Uncooked, mg.	Cooked, mg.	Uncooked, mg.	Cooked, mg.	Uncooked, mg.	Cooked, mg.	Uncooked, mg.	Cooked, mg.
10	9.02	18.92	14.21	19.21	8.20	21.41	18.11	23.24
20	14.50	29.73	27.31	32.14	22.13	37.65	30.65	39.10
30	20.71	36.44	36.00	40.01	34.26	44.02	42.16	46.72
60	24.32	53.60	45.36	60.71	46.72	58.91	50.01	64.51
120	32.67	58.12	50.05	73.44	52.8	68.41	53.25	81.63
180	35.46	59.01	52.78	76.89	53.75	71.24	55.43	88.01

TABLE IX.

Digestibility of various starches by pancreatic amylase (in vitro).
(Reducing sugar is expressed as mg. of maltose.)

Time in minutes.	TAPIOCA.		RICE.		WHEAT.		POTATO.	
	Uncooked, mg.	Cooked, mg.	Uncooked, mg.	Cooked, mg.	Uncooked, mg.	Cooked, mg.	Uncooked, mg.	Cooked, mg.
10	2.98	20.14	19.47	29.92	6.97	25.25	15.21	32.73
20	9.00	29.43	39.10	41.61	19.70	34.71	28.30	49.34
30	18.01	32.95	45.04	50.05	33.04	41.46	36.00	58.10
60	25.20	41.62	50.01	58.24	44.00	49.34	47.60	71.17
120	26.96	45.05	53.20	62.40	52.70	55.63	49.52	84.25
180	29.04	47.37	55.10	64.57	54.30	58.51	52.70	92.91

TABLE X.

Digestibility of the carbohydrates of tapioca per cent.

	Moisture.	Protein.	Fat.	Ash.	Fibre.	Glucose.	Fructose.	Sucrose.	Starch.	Total digestible carbohydrate.	Total carbohydrate by difference.	Digestibility.
Tapioca (raw) ...	21.25	1.863	0.32	1.565	2.2	0.189	0.20	0.08	34.7	35.169	72.8	48.3
„ (cooked, calculated on 21.24 per cent moisture basis).	0.21	0.19	0.11	56.2	56.71	...	77.9

Experiments carried out on the carbohydrates of tapioca show that they are hydrolysed by amylase to an extent of 48 per cent in raw state and 78 per cent after cooking. *In vitro* experiments carried out on human beings by Langworthy and Devel (1922) show that the cassava and potato starches are digested almost completely. It is to be noted, however, that the *in vitro* experiments described in this paper were carried out with a single enzyme, and the conditions did not correspond with those in the digestive system where carbohydrates are subjected to the cumulative action of various enzymes and other hydrolysing agents. The observations of Langworthy and Devel (1920) show that relatively large quantities of raw potato or raw corn starch can be assimilated by normal human subjects. They reported that raw potato starch was the 'less digestible' of the two. Beazell, Schmidt and Ivy (1939) found in the course of *in vitro* experiments that raw potato starch was almost entirely refractory to the action of the gastro-intestinal enzymes. According to these authors the disappearance of starch during its passage through the alimentary tract does not necessarily mean that all that has disappeared has been digested and assimilated, for bacterial fermentation accounts for the decomposition of a major part of the starch.

In vitro digestion experiments conducted on the raw starches show that, in general, the starches are more slowly acted upon by digestive enzymes in the raw state than when cooked. It is known that cooking improves the digestibility of starch to a less extent than that of protein. The simultaneous action of a proteolytic enzyme (trypsin) and a diastase causes more rapid digestion of starch and protein than diastase or protease acting alone (Carman, Smith, Havens and Murtin, 1930).

The above results show that the carbohydrates of tapioca are more easily attacked by taka diastase than by pancreatic amylase. A similar observation has been made by Basu and Mukherjee (1936) during their work on the digestibility of starches from different varieties of Bengal rice. This is, however, of little practical importance since taka diastase is an enzyme found only in fungi and not in the human digestive system.

SUMMARY.

1. The nitrogen complex of tapioca (*Manihot utilissima*) was investigated. A major portion of the nitrogen exists in the form of simple nitrogenous compounds. An attempt was made to isolate the proteins of the root in pure form.

2. Both the protein and non-protein-fraction contain the amino-acids, tyrosine, tryptophane and cystine in fair amounts and have a high arginine content.

3. *In vitro* digestibility experiments showed that the digestibility of tapioca proteins was not inferior to that of rice protein.

4. The chief defect of tapioca as a source of protein appears to lie in its low total protein content rather than in the quality of the protein present.

5. The digestibility of the carbohydrates of tapioca by two enzymes has been studied and found to be 48.3 per cent in raw state and 77.9 per cent after cooking. The starch is digested to a greater extent by taka diastase than by pancreatic amylase.

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AN OUTBREAK OF LATHYRISM IN CENTRAL INDIA.

BY

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LATHYRISM is an ancient disease. According to Chopra (1935) 'in the old Hindu literature, Bhavaprokasa, it is written that "the tripura pulse" causes a man to become lame and crippled and it irritates the nerves'. Hippocrates observed that the prolonged use of certain peas as food was liable to cause paralysis. In the seventeenth and eighteenth centuries outbreaks of disease which were ascribed to the eating of lathyrus occurred in France, Germany, Italy and Algiers. The first account of lathyrism was written by a layman, General Sleeman, over 100 years ago. Sleeman (1844) encountered an outbreak in a number of villages near Saugor in the Central Provinces in 1834-36 and described it in his 'Rambles and Recollections of an Indian Official', published in 1844. His account, which is clear and vivid and could scarcely be bettered to-day, may with advantage be quoted in full:—

'In 1829 the wheat and other spring crops in Saugor and surrounding villages were destroyed by severe hailstorms and rains and in 1831 they were destroyed by blight. During these three years the "teori" or what in other parts of India is called "kesari" (*Lathyrus sativus*), a kind of wild vetch which, though not sown itself, is left carelessly to grow among the wheat and other grains and given in the green and dry state to cattle, remained uninjured and thrived with great luxuriance. In 1831 they reaped a rich crop of it from the blighted wheat fields and subsisted upon its grain during that and the following years, giving the stalks and leaves only to their cattle. In 1833 the sad effects of this food began to manifest themselves. The younger part of the population of this and the surrounding villages, from the age of thirty downwards, began to be deprived of the use of their limbs below the waist by paralytic strokes, in all cases sudden, but in some cases more severe than others. About half the youth of this village of both sexes became affected during the year 1833-34 and many of them have lost the use of their lower limbs entirely and are unable to move. The youth of the surrounding villages in which the "teori" from the same causes formed the chief article of diet during the years 1831-32 have suffered to an equal degree. Since the year 1834 no new case has occurred but no person once attacked had been found to recover the use of limbs affected and my tent was surrounded by great numbers of the youth, in different stages of the disease, imploring my advice and assistance under the dreadful visitation. Some of them were very fine young men of good caste and respectable families and all stated that their pains and infirmities were confined entirely to the part below the waist. They described the attack as coming on suddenly, often while the person was asleep, and without any warning symptoms whatever and stated, that a greater portion of young men were attacked than of the young women. It is the prevailing opinion of the natives throughout the country that both horses and bullocks, which have been much fed upon "teori", are liable to lose the use of their limbs, but if the poisonous quantities abound more in grain than the stalk or leaves, a man who eats nothing but the grain must be more liable to suffer from the use of the food than beasts, which eat it merely as they eat grass or hay.'

In more recent times lathyrism has occurred in India in epidemic form in the Central Provinces (Buchanan, 1904), in Rewa, Central India Agency (Acton, 1922; McCombie Young, 1927), in Gilgit (McCarrison, 1926; Mackenzie, 1927), in the United Provinces (Stott, 1930) and in the Punjab (Shah, 1939). Megaw and Gupta (1927) concluded from information supplied by civil surgeons in response to a questionnaire that the disease is mainly confined to a belt which runs across the Central Provinces, the east of the United Provinces and the north of Bihar. It also occurs in certain restricted areas in the Punjab and the United Provinces outside this belt. Lathyrism appears to be unknown in South India. Minchin (1940) reported a series of cases of spastic paralysis of the lower limbs in the Madras General Hospital which he considered to be clinically indistinguishable from lathyrism, but there was no evidence that lathyrus seeds had formed part of the diet of the patients.

The epidemic described in this paper occurred in Bhopal State in Central India in 1944-45. At the Fifth All-India Food Conference held in Delhi at the end of January 1945, an official of that state consulted Dr. W. R. Aykroyd, Director of Nutrition Research, then serving on the Famine Inquiry Commission, about an outbreak of disease which the people affected ascribed to the consumption of a certain kind of pulse. The account given obviously suggested lathyrism. A few days later the author was sent to Bhopal to investigate the outbreak.

One of the conclusions reached in the investigation is that lathyrism, as its name implies, is due to eating lathyrus. There have been other theories of origin, which will be referred to later. As regards the immediate cause of the disease, nothing has been discovered which does not conform to the observations of Sleeman and the classical conception of ætiology. An attempt has, however, been made to study the social, economic and agricultural background of the outbreak—to discover, in fact, *why* the victims of the disease were forced to rely on lathyrus as their staple food. Accordingly, before the clinical features of the epidemic are described, an account will be given of the environment in which it occurred.

Bhopal State, which has an area of 6,902 square miles and a population (1941) of 785,000, is divided for administrative purposes into two districts, eastern and western. It was in the eastern district that lathyrism made its appearance. This district is an upland area, partially covered with forests, about 1,000 to 1,500 feet above sea-level, and less fertile than the western district. The population is mainly Hindu. Malaria is prevalent and the spleen rate is high.

Except in Bhopal City and certain smaller towns, the entire population is engaged in agriculture and lives on food produced in the area. There are three types of workers in the villages :—

- (a) land-owning cultivators ;
- (b) sub-tenants of the above ;
- (c) labourers—these do various kinds of work as well as cultivation. Labourers are usually paid in kind and very rarely in cash.

There are no modern means of irrigation. In bad seasons, rust, blight, hailstorms, excess of rain, etc., may lead to danger of scarcity and food shortage. The diet changes to some extent with the season, following the succession of harvests.

The principal food crops and seasons of sowing and harvesting are as follows :—

Crop.			Time of sowing.	Time of harvesting.
Rice	June	October
Maize	"	December
'Jowar' (<i>Sorghum vulgare</i>)	"	"
'Sawan' (<i>Panicum frumentaceum</i>)	"	October
'Kodon' (<i>Paspalum stoloniferum</i>)	"	"
Black gram (<i>Phaseolus mungo</i>)	"	"
Red gram (<i>Cajanus indicus</i>)	"	"
Wheat	October-November	March
Barley (<i>Hordeum vulgare</i>)	October	"
Bengal gram (<i>Cicer arietinum</i>)	"	February-March
Lentil (<i>Lens esculenta</i>)	"	"
'Teora' (<i>Lathyrus sativus</i>)	"	"

Lathyrus sativus is known as 'teora' or 'teori' in this part of India. Various names including 'matra', 'matri', 'batra', 'khesari', 'garash', etc., are used in other parts of

the country. Howard and Khan (1928) described in detail the Indian types of *Lathyrus sativus*. It is an important crop in the eastern district of Bhopal for the following reasons :—

1. It is used as a fodder crop.
2. It gives a good yield with minimum labour. It is a hardy plant and thrives well in unfavourable circumstances.
3. It is a stand-by to the cultivator should there be a failure of wheat supplies. If wheat is in short supply 'teora' grains are consumed in greater quantities.

Vegetables are grown in only a few villages and on a very limited scale. Vegetable sellers called 'kachhis' may come once or twice a month to sell vegetables to those who can afford them. The following vegetables are grown in the district :—

Spinach	Bitter gourd
Onion	Carrots
Brinjal	Tomatoes
Cucumber	Radish

Bengal gram leaves	These are eaten frequently.
'Rajgra' leaves	These are wild plants, taken occasionally but not relished by the villagers.
'Bathua' leaves	

Fruits.—There are very few fruit gardens in the districts and the poor rural community cannot afford to buy imported fruits. Mangoes, papaya, 'ber' (plums—*Zizyphus jojoba*), 'mohwa' and 'achar' are consumed to some small extent in the district.

Milk and milk products.—Milk is obtained both from cows and buffaloes. The cattle are small and degenerate though grazing grounds are abundant in the area. The average cow in the area yields about 1 lb. to 1½ lb. of milk per day, and an average buffalo 3 lb. to 5 lb. In Table I the population of human beings and the number of milch cattle in two villages in the area are shown :—

TABLE I.

Name of village.				Population.	Number of cows.	Number of buffaloes.
Birpur	940	110	69
Umarkhoe	430	62	29

Even the small quantity of available milk is not usually consumed by those who keep cattle. The milk is converted into curds and the milk fat is removed. The ghee is sold, and the buttermilk is consumed.

Meat and eggs.—Tradition and taboo prevent the eating of these foods. The great majority of the population is Hindu and does not take meat at all, while the few Mohammedans take meat very rarely.

Sugar.—Little sugarcane is grown in this area and the consumption of sugar is almost negligible.

The preparation and cooking of food.—The whole grains (both cereals and pulses) are ground into flour ('atta') and this flour is usually made into 'chapatties'. Whole or coarsely crushed pulses are boiled with water and consumed as porridge after the addition of salt and condiments.

Conditions prevailing in the area at the time of the outbreak of lathyrism.—During the last 2 to 3 years, crops, especially wheat, in this area have been damaged by rust, blight, hailstorms, etc. The hardy 'teora', however, thrived. While wheat forms the staple diet of the population, the poor mix cheaper grains with wheat. Failure of the wheat crop had the

result that the poor consumed cheaper grains in increasing quantity. The prices of food grains have risen to 2 to 3 times the pre-war level. In Table II the market rates of different food grains for the period 1939 to 1945 are shown :—

TABLE II.

Market rates at which different food grains were available in the period from 1939 to 1945 (per 5 maunds).

Name of foodstuff.				1939.	1940.	1941.	1942.	1943.	1944.	1945.
				Rs.	Rs.	Rs.	Rs.	Rs.	Rs.	Rs.
Wheat	12	16	16	20	24 to 30	30	30
'Teora'	8	12	12	13	20	20	20
Bengal gram	10	14	14	18	25	25	25
Lentil	12	18	10	20	25	25	25
'Batla'*	8	12	13	15	20	20	20

* 'Batla' is a pulse which resembles a pea. Its consumption is being encouraged as an alternative to 'teora'.

In 1944 the state authorities introduced the rationing of wheat into the urban areas. To meet the demand of the towns the cultivators were asked to give on payment certain quantities of wheat per acre sown. In areas where the wheat crop was poor, the cultivator was left with limited supplies of wheat. The position of agricultural labourers was also affected for the following reasons: (a) The labourers are paid in kind. Cultivators did not have enough wheat and the labourers were given 'teora' (*Lathyrus sativus*) instead of wheat. (b) The landowning cultivators found a ready excuse for refusing demands for wheat on the part of their employees by saying that practically all their wheat had been taken by the state and that nothing was left. The net result of the crop failure and the procurement operations was that the poorer section of community was reduced to living almost entirely on 'teora'. Visits to houses in villages at the time of preparing meals confirmed the above statement. It was found that poor villagers generally consumed 'chapatties' made of 'teora'. In some cases 'teora' was mixed with Bengal gram, barley or wheat.

State of nutrition.—One hundred children in a village were examined for the incidence of clinical signs of deficiency diseases. The results obtained are as follows :—

Number of children showing phrynoderma—7 per cent.

„ „ „ Bitot's spots—Nil.

„ „ „ angular stomatitis—2 per cent.

Practically all the children showed discoloration of the conjunctivæ. The general impression gained from this survey was that the state of nutrition was very similar to that of village children in many other parts of the country. This is a point of importance, since suggestions have been made that vitamin deficiency plays a part in the causation of lathyrism.

THE OUTBREAK.

Preliminary inquiries revealed that at least 1,200 cases had occurred in the district. Fifteen villages in three tahsils in which a high incidence was reported were visited and about 150 people showing evidence of the disease were examined. A full history of 75 cases of recent onset during the second half of 1944 or early in 1945 was obtained. It was felt that cases occurring earlier might not be able to recollect details about the onset of the disease and attention was focused on more recent cases.

The disease is not a mysterious unfamiliar malady to the inhabitants of this area. Every year, or every second or third year, a few persons contract it. Villagers are familiar with it and know that it is associated with famine and poverty, and that it is not an infectious disease. It may be added that the present outbreak of lathyrism was not confined to Bhopal State. There were reports of cases occurring in adjacent areas outside the state boundaries.

Age.—The villagers cannot give their age with any degree of exactness. Table III shows approximately the incidence of the disease by age groups:—

TABLE III.

Age group, years.	Number of cases.	Percentage.
4-10	13	18.0
11-20	19	28.4
21-30	21	29.1
31-40	11	15.2
41-50	7	9.7
51-60	1	1.3

Buchanan (*loc. cit.*), McCombie Young (*loc. cit.*) and Shah (*loc. cit.*) all observed that the majority of cases were in the age groups 11-20 and 21-30. This agrees with the observed age incidence in the present investigation.

Season.—Table IV gives the number of cases and the percentage of cases occurring in different months:—

TABLE IV.

Month of onset.	Number of cases.	Percentage.
January	2	2.9
February	1	1.5
March
April
May
June
July
August	11	15.9
September	23	33.9
October	22	31.8
November	9	13.0
December	1	1.5
TOTAL	69	...

The above figures show that most of the cases occurred in the months of August, September and October, the highest number being in the month of September. Shah (*loc. cit.*) recorded that most of his cases occurred in August and McCombie Young (*loc. cit.*) observed a peak in October. It is during or just after the rainy season that the paralysis of lathyrism tends to appear.

Sex incidence.—The percentage of cases occurring in the population of two villages and the sex incidence are shown in Table V:—

TABLE V.

Name of village.	Total population.	Sex.	Number of persons.	Number of persons suffering from lathyrism.	Percentage.
Birpur	940	M	513	37	7.2
		F	427	5	1.1
Umakhoh	430	M	223	23	10.3
		F	207	4	1.9

The above shows that males suffer more than females. Out of the 69 persons suffering from the disease in these villages, only 9 were females. This is in conformity with the findings of the earlier workers previously referred to.

Economic status.—The victims of lathyrism belonged to the poorest section of the community, usually labourers in villages owning no land. Out of the 73 persons afflicted whose history was taken only 4 were cultivators owning some small parcel of land, and there was one 'Bania' (grocer). The others were all labourers. 'Chamars' (shoe-makers) contributed the largest number of victims.

Incidence of signs of diet-deficiency diseases.—All the cases were examined for clinical signs of dietary-deficiency diseases. None showed angular stomatitis. There were 3 cases of phrynodema among the 73 cases. A history of night-blindness was given by 2 cases. The general impression was gained that the affected persons were not badly nourished.

Clinical findings. (a) *Onset.*—In all the patients examined, no premonitory symptoms such as an attack of nausea, vomiting after meals, or diarrhoea, were complained of. McCarrison (1926) noted that constipation tended to occur in his cases, but this was not a prominent feature in the present series. Of the 73 persons examined, only 10 gave a history of mild or severe constipation. Inquiries revealed that 15 cases suffered, previous to the onset of the disease, from fever which was most probably malaria.

In 33 patients the onset of the disease was sudden. Some gave a history of sleeping normally and awakening with stiffness of the muscles and partial loss of control of the lower limbs. Others fell down while working (ploughing, etc.), and found themselves unable to get up and walk back to their houses. In the remaining 40 cases the onset of the disease was slower. In one case the patient did not know that he was a sufferer until others pointed it out to him after seeing him walking with bent knees. A few days later he found himself paralysed. In cases in which the onset was slow the patients first suffered from backache and stiffness of the lower extremities with some difficulty in dorsiflexion of the knees. Severer signs and symptoms followed slowly. Some patients reported that in the early stages contraction of the muscles of the calves had occurred. A 'lump would form' in the morning when the patient was getting out of bed. These 'lumps' of contracted muscles would prevent the straightening of the lower extremities. The patients would massage these out and for the rest of the day could walk without much difficulty. Then slowly severer symptoms would set in.

(b) *Gait*.—In mild cases there was only a little bending of the knees and patients felt difficulty only on running or on going downhill. In more severe cases the patients walked with bent knees, the knees being approximated and the feet more widely separated than normal. In this stage the heels leave the ground earlier than the toes and there is some swaying of the hips. In more advanced cases the 'scissors' gait (crossing of the legs on walking) developed, with a tendency on the part of the patient to walk on the toes and swaying of the hips was more marked. Such patients required the use of one or two sticks, according to the severity of the paralysis. Very severely affected patients were reduced to crawling in a sitting position. In all the above stages instability was increased by running, jumping, crossing a small drain, going down a hill, etc. There was a frequent tendency on the part of patients to fall down.

(c) *Musculature*.—There was no marked change in the appearance of the muscles of the lower extremities. Muscular tone was, in most cases, in excess of normal. Spasm of the calf and thigh muscles occurred on slight exertion or in the absence of exertion in some cases. No case complained of any weakness in the upper extremities.

(d) *Reflexes*.—In 63 cases the epigastric and abdominal reflexes were exaggerated. The plantar reflex was extensor in type in those in which it could be elicited. The knee-jerks were exaggerated in all cases. Ankle-clonus was present in 62 cases. The sphincters of the rectum and the bladder were unaffected.

(e) *Sensation* was normal in all cases. There was no change in the functioning of the cranial nerves. Speech, sight, smell, hearing, etc., were normal. Mental symptoms and psychological disturbances were not observed.

It is apparent that the paralysis of lathyrism is an upper motor neuron lesion. Presumably the pyramidal tracts are involved. No post-mortem histological examination of the spinal cord in cases of lathyrism appears to be on record in the literature.

ÆTIOLOGY.

Villagers in the area affected by the outbreak and the victims of the disease have no doubt that the paralysis is caused by consuming lathyrus seeds in large quantities for several months. This is the traditional view of the cause of the disease; hence, in fact, its name. The observations of Sleeman have already been quoted. A number of those who have studied the disease in India (Buchanan, *loc. cit.*; McCarrison, 1928; McCombie Young, *loc. cit.*, and others) have adopted the traditional standpoint. Anderson, Howard and Simonsen (1925) and Shah (*loc. cit.*), however, consider that another pulse, *Vicia sativa*, may be the cause of the disease.

The feeding of *Lathyrus sativus* or *Vicia sativa* to rats by McCarrison (1928) and to ponies by Stott (*loc. cit.*) did not result in the production of lathyrism or of any lesions of the nervous system.

During the present investigation it was found that those who developed lathyrism had usually consumed *Lathyrus sativus* in large quantities for six months or more. It was impossible to obtain exact information about the proportion of *Lathyrus sativus* in their diet. It appears, however, that it formed the great bulk of the diet. The victims of the disease were mostly labourers, and were paid in kind. They ate what they could get, and this was mainly lathyrus seeds. It was said by the villagers that those who mixed *Lathyrus sativus* grains with equal amounts of wheat did not contract lathyrism.

Anderson *et al.* (*loc. cit.*) stated that samples of 'khesari dhal' or lathyrus obtained from localities in India in which outbreaks of lathyrism had occurred were contaminated with the vetch *Vicia sativa*, popularly known as 'akta'. They reported that 'akta' was poisonous to ducks and monkeys whereas lathyrus was not. The toxic signs produced in these species by the feeding of diets containing 'akta' did not, however, closely resemble human lathyrism. In the present inquiry the question whether the lathyrus seeds which were being consumed in the affected district were contaminated with 'akta' was investigated. A pure sample of *Vicia sativa* was obtained from Dr. B. P. Pal of the Imperial Agricultural Research Institute, New Delhi, and was shown to stockists in villages and towns, and to intelligent cultivators

in various villages. In not a single case did they recognize the *Vicia sativa* seeds and all were sure that such seeds were not to be found in *Lathyrus sativus* stocks. About 50 samples of *Lathyrus sativus* were collected from various families and in various villages. Some of those were from stocks actually consumed by people who developed lathyrism. Careful examination of these samples in the Imperial Agricultural Research Institute, New Delhi, failed to reveal the presence of *Vicia sativa* seeds.

At three places about 200 lb. of *Lathyrus sativus* were sifted through a sieve with a mesh smaller than normal *Lathyrus sativus* grain. A search was made in the sieved material for 'akta' seeds, which are smaller than lathyrus seeds. No 'akta' could, however, be sieved out in this way.

It was thought that 'akta' seeds, being lighter and smaller than *Lathyrus sativus* seeds, might be separated with the chaff in the process of winnowing. A sample was taken from the latest crop in the field which was ready for separation. The husk was carefully removed. The resultant seeds were examined for 'akta' seeds but none could be found. This shows that *Lathyrus sativus* stocks were not contaminated with *Vicia sativa*.

Mellanby (1934) has put forward the theory that 'lathyrism is due to an active neurotoxin in the food whose detrimental effects can be prevented by protective foods containing vitamin A and carotene, such as milk, eggs and green vegetables, even when much of the toxic agent in the lathyrus peas is consumed. It may therefore be classed among the deficiency diseases'. McCombie Young (*loc. cit.*) was also of the opinion that vitamin A deficiency was concerned in causation. Lesions of the nervous system have, however, been more frequently observed in experimental animals as a result of deficiency of various members of the vitamin B₂ group than as a result of deficiency of vitamin A. The present investigation has not thrown any further light on the problem whether lack of vitamins is associated with a toxic factor in producing the paralysis. Lathyrism is a famine disease and the circumstances which lead to an increased intake of lathyrus will at the same time lead to a decreased intake of other foods, including protective foods. None of the facts revealed is incompatible with Mellanby's hypothesis, but in view of the low incidence of vitamin deficiency states in the affected area, the author is of the opinion that lathyrism should be regarded as a disease due to a toxin of unknown nature rather than as a deficiency disease. Until, however, lathyrism has been satisfactorily reproduced in experimental animals, the question as to what part vitamin deficiency may play in causation cannot be finally answered.

The higher incidence of the disease amongst men than women can probably be explained by the greater intake of lathyrus on the part of the former. During the rainy season and just after, agricultural labourers work hard at ploughing and other agricultural operations. Their calorie requirements are correspondingly increased and when only lathyrus is available to fulfil these they eat it in large quantities.

TREATMENT AND PREVENTION.

As far as at present known, the nervous lesions are permanent and the disease incurable. No social study has been made of the fate of the victims of lathyrism; many no doubt remain in their villages and contribute to the work of the village as far as their crippled state permits; others swell the ranks of beggars in cities. It has been stated that many of the paralysed beggars who obstruct the pavements of Calcutta and other large cities are victims of lathyrism.

Prevention is a social, economic, administrative and agricultural problem. Lathyrus seeds can apparently be safely consumed in small quantities, but when they form the bulk of the diet the disease makes its appearance. The circumstances in which this tends to occur have been described in this paper. Satisfactory preventive measures include encouraging the production of suitable alternative crops to lathyrus, the improvement of the economic status of agricultural labourers, and the importation of other foods such as wheat into endemic areas when an outbreak threatens. A watch should be kept by public health authorities for the appearance of the disease in such areas in Central India and elsewhere in the country.

Procurement operations to supply wheat to towns under rationing schemes should not be allowed to deprive rural areas, in which lathyrus is a stand-by, of excessive amounts of wheat.

SUMMARY.

1. An outbreak of lathyrism in Central India has been investigated. This was associated with the consumption of *Lathyrus sativus* in large quantities for a period of six months or more. Some 1,200 cases occurred in the district. Of these, 150 were seen, and full notes of 73 cases were taken.

2. Villagers in the area in question and patients who contracted the disease realized that it was due to the consumption of this pulse. They also realized that it is not an infectious disease and understood its association with poverty.

3. The chief sufferers from lathyrism were young adult males belonging to the poorest classes. September and October were the months of greatest prevalence.

4. Clinically the disease manifests itself as a spastic paralysis of the lower limbs. The spinoteres of the bladder and the rectum are not affected. No mental or sensory disturbances were observed.

5. No *Vicia sativa* seeds were found in the stocks of lathyrus seeds consumed in the villages in which the outbreak occurred. The investigation points to the existence in *Lathyrus sativus* of a toxin affecting the pyramidal tracts.

6. Preventive measures are discussed.

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NUTRITION AND DIETARY SURVEYS IN BIJAPUR DISTRICT (BOMBAY PRESIDENCY), AT THE END OF THE FAMINE YEAR OF 1943.

BY

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BIJAPUR DISTRICT is situated at the southern end of the Bombay Presidency and adjoins Hyderabad State. It is served by both the monsoons. but in fact gets only the tail-end of both, with the result that rainfall in the district is extremely irregular both in amount and distribution. The average annual rainfall is 21 inches. The whole district is liable to famine.

CROPS AND DIET.

The staple diet of the people is 'jowar' (*Sorghum vulgare*), but 'bajri' (*Pennisetum typhoideum*) is also consumed to some extent, particularly in the cold season. Rice is consumed in negligible quantities and its use is mainly confined to town people. Wheat is also consumed in small amounts in villages, mostly on religious occasions.

'Jowar' and 'bajri' are the main food crops grown in the district. Other major commercial crops are ground-nut and linseed. The population of the district according to the 1941 Census was 975,982, which, by the end of 1943, the period in which this survey was carried out, may have reached a little over a million. The area under food crops exceeds 2.4 million acres and hence the cultivated area under such crops works out to nearly 2.5 acres *per capita*.

FAMINE CONDITIONS AND RELIEF MEASURES.

Not infrequently the rains fail and famine follows. This occurred in 1942, when early rains were sparse, while late September rains, which are essential both for *kharif* as also for *rabbi* (winter) crops, failed completely with the result that *kharif* crop withered away and the *rabbi* crop did not grow. From the beginning of the year 1943, famine conditions prevailed in the whole district.

Shortage of cereals began to be experienced from the early part of 1943 and imports were made from neighbouring surplus districts. The available foodstuffs were equitably rationed among the people. About 100,000 people left the district in the early part of the year to other more affluent adjoining areas for work. Extensive relief measures were started in the district itself; more than 100,000 people were employed on famine works, such as the construction of tanks, roads, bunding works, etc. Seventy thousand people who were unable to work were given doles, while 18,000 persons, mainly women and children, were fed at free kitchens opened by charitable committees.

The months of June, July, August and September were trying ones. During these months green vegetables were not available, and milk and milk products were very scarce. The diet of the village people consisted only of rationed cereals, supplemented occasionally with a small quantity of pulses, and a little red chillies, onion or garlic being used as a condiment. However, during 1943, there were very good early rains and the late September rains were also good. With the arrival of the new crop the situation was relieved by the end of October and the famine was officially declared as over by the end of November 1943.

Table I gives the vital statistics in the district for 5 years including the famine year of 1943. There was a fall in the total number of births during the year probably due to

migration. There was an increase in the infant mortality rate. The crude general death rate showed a slight decrease but in this connection the large numbers emigrating must be taken into consideration. There was a slight increase in the deaths among children of the age groups 1 to 5 and 6 to 10. The mortality from cholera and smallpox showed a slight increase over that for the previous year, but these diseases were kept under control by appropriate measures.

TABLE I.
Vital statistics in Bijapur district.

Year.	Total births and (a) crude rate per mille.	Total deaths and (b) crude rate per mille.	Deaths under 1 year and (c) infant mor- tality rate.	1 to 5 years.	6 to 10 years.	Over 60.	Dysentery.	Diarrhœa.	Tuberculosis.	Other respiratory diseases.	Cholera.	Smallpox.	Plague.
1	2	3	4	5	6	7	8	9	10	11	12	13	14
1940	... { 37,549 (a) 43.19	27,571 (b) 31.72	5,478 (c) 144.8	5,632	1,706	5,289	77	1,274	751	3,160	6	56	2,434
1941	... { 40,162 (a) 41.15	27,426 (b) 28.10	5,350 (c) 133.2	6,249	1,609	5,325	97	1,870	896	3,216	1,276	63	957
1942	... { 37,192 (a) 39.13	25,229 (b) 25.85	5,323 (c) 139.3	5,924	1,655	4,926	27	1,817	827	2,943	1,167	137	16
1943	... { 32,753 (a) 33.52	24,929 (b) 24.8	4,866 (c) 143.4	6,129	1,717	3,057	44	1,325	768	2,878	1,332	363	1
January to July 1944.	18,505	13,007	2,421	3,081	960	2,960	13	515	421	1,850	2	52	...

On the whole the vital statistics show that violent ill effects were not produced by the famine; this reflects to some extent the beneficial effects of the extensive relief measures and the preventive anti-epidemic measures organized in the district.

DIETARY SURVEY.

A dietary survey of 42 families in 4 different villages in 3 talukas was carried out during the month of December 1943. By this time the new bumper *kharif* crop was already in consumption. Families were divided into 4 groups according to their annual income and occupation and according to whether they were vegetarian or non-vegetarian:—

GROUP I : Consisted of poor families of daily labourers on fields who are usually paid in kind. Annual income below Rs. 100.

GROUP II : Consisted of small agriculturists engaged in casual labour. It also included those who cultivated land on a rental basis and village artisans. Annual income above Rs. 100 but below Rs. 300.

GROUP III : Consisted of landowners depending on their own land. It also included village teachers, small traders, etc. Annual income above Rs. 300 but below Rs. 500.

GROUP IV : Consisted of big landowners, big village merchants, etc., with incomes above Rs. 500 but below Rs. 2,000.

The dietary survey was carried out for 10 days in each village. The average intake of various foodstuffs on the part of families per consumption unit (C. U.) per day for the different income groups is given in Table II :—

TABLE II.

Average intake of various foods in ounces per consumption unit per day by income groups in 42 families surveyed in Bijapur district in December 1943.

GROUP :—	VEGETARIAN.				NON-VEGETARIAN.	
	I	II	III	IV	I	II
Number of families :—	7	17	4	3	5	6
1. Total cereals ...	19·7	28·8	27·8	23·35	24·2	26·0
2. 'Bajri' ...	10·55	16·6	17·5	13·81	18·05	6·15
3. 'Jowar' ...	8·94	10·6	7·0	4·1	11·46	14·50
4. Rice ...	0·02	0·18	1·08	1·2	<i>Nil</i>	<i>Nil</i>
5. Wheat ...	0·36	1·3	2·1	3·86	<i>Nil</i>	<i>Nil</i>
6. <i>Navani</i> (Italian millet) ...	0·7	<i>Nil</i>	<i>Nil</i>	0·2	<i>Nil</i>	<i>Nil</i>
7. Pulses ...	0·79	1·28	1·81	1·4	0·59	1·33
8. Green leafy vegetables ...	1·5	0·81	1·29	2·89	2·1	1·36
9. Non-leafy vegetables ...	1·32	1·16	2·35	1·43	0·72	2·4
10. Ground-nut ...	2·78	<i>Nil</i>	0·23	<i>Nil</i>	0·26	<i>Nil</i>
11. Milk and milk products ...	0·3	1·07	2·88	3·7	0·17	0·85
12. Butter ...	0·01	0·06	0·23	0·3	<i>Nil</i>	0·06
13. Vegetable oil ...	0·53	0·2	0·38	0·99	0·2	0·19
14. Sugar and jaggery ...	0·35	0·74	0·64	1·03	0·24	<i>Nil</i>
15. <i>Condiments</i> : green and red chillies.	1·29	1·3	0·78	2·11	1·8	2·2
16. Meat ...	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	1·78	1·08
17. Fish ...	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	<i>Nil</i>	0·1	0·2

families. Thus, 38 per cent of families had less than 1,000 I. U. of vitamin A per C. U. (*vide* Table II-b).

In one typical village family members surveyed were examined for deficiency diseases with the following result (Table III):—

TABLE III.
Deficiency disease noted.

Number of persons examined.	Xerosis.	Bitot's spots.	Night blindness.	Phrynoderma.	Bleeding gums.	Angular stomatitis.	Rickets.
59	21	9	1	5	3	Nil	1
	(35·5 per cent)	(15·2 per cent)	(1·6 per cent)	(8·4 per cent)	(5 per cent)	...	(1·6 per cent)

Vitamin A deficiency appeared to be common in the families examined. Some degree of vitamin C deficiency was noticed, as also a case of rickets.

INCIDENCE OF DEFICIENCY DISEASES IN SCHOOL CHILDREN.

In all 5,131 children were examined in schools in different talukas in the district for nutritional deficiencies. They were classified in the same income groups as was done above, the criterion being the income of the families to which they belong. Table IV gives the incidence of deficiency diseases among children by income groups.

TABLE IV.
Percentage incidence of deficiency diseases in school children by income groups in Bijapur district.

Group.	Number of children examined.	Xerosis.	Bitot's spots.	Angular stomatitis.	Bleeding gums.	Phrynoderma.
I	2,485	41·1	5·3	1·0	4·9	7·3
II	1,576	31·8	4·7	0·9	4·6	4·5
III	686	25·6	2·9	1·0	2·9	2·7
IV	384	21·3	3·3	1·5	3·1	1·5
TOTALS ...	5,131	35·7	4·8	1·1	4·7	5·4

It will be seen that vitamin A deficiency as indicated by xerosis and Bitot's spots was highly prevalent in all the groups. This was far above the normal if figures revealing in a survey in an adjoining area in Hyderabad (Deccan) State are taken for purpose of comparison (Daver and Ahmad, 1942). The incidence of vitamin C deficiency, as indicated by bleeding

gums, was also much above the normal. Due to severe famine conditions prevailing, very little green vegetables were available during the year. Similarly, milk and milk products were also very scarce.

However, cases of softening of cornea and phthisis bulbi indicating gross effect of vitamin A deficiency were rarely seen. Cases of gross scurvy were also few. No cases of nutritional oedema, beri-beri or pellagra were seen. This may be due to the fact that the staple diet of the people is *wholly* millets and not rice. Angular stomatitis was present to a slight extent. Sore-mouth was not observed, and children showing angular stomatitis had no other mouth lesion in most cases. A similar observation was reported by Wilson and Widdowson (1942) in wheat and millet-growing areas. Cases of infantile rickets were seen both during the years 1942 and 1943, during the routine vaccination inspection of children at some villages. It seems that rickets is not uncommon in this district, though the incidence may not be high.

Table IV indicates that deficiencies among school children were on the whole higher in lower income groups. In fact it was 50 per cent greater in the lowest income group (I) over that in the highest income group (IV).

TABLE IV-a.

Statement of some important diseases treated at the hospitals and dispensaries in Bijapur district.

Total number of patients treated for	YEAR.		
	1941.	1942.	1943.
1. All diseases	136,859	121,514	143,198
2. Malaria	18,125	13,449	17,957
3. Diarrhoea	2,917	2,928	3,016
4. Typhoid (enteric fever)	205	27	362
5. Dysentery (amœbic and bacillary)	2,351	2,931	3,403
6. Pneumonia	403	430	416
7. Influenza	2,705	2,705	3,531
8. Tuberculosis	351	294	233
9. Rickets	186	194	144
10. Scurvy	20	11	26
11. Beri-beri	1	3	...
12. Xerophthalmia	1	4	2
13. Epidemic dropsy	1	...

Table IV-a gives the number of persons treated for various diseases, including deficiency disease, at the taluka dispensaries in the district during the years 1941, 1942 and 1943. The incidence of rickets in this district is different from that observed for the adjoining millet-growing area of Hyderabad (Deccan) where it was stated to be *nil* by Wilson and Widdowson (*loc. cit.*).

All the children seen for nutritional deficiencies were also examined clinically for assessment of the state of nutrition. Their height in inches and weight in pounds were also noted for different age groups. The means of 3,298 of these are given in Table V. The average of height and weight appear to be similar to those of under-nourished children of the poorer classes elsewhere in India.

The results of clinical examination are given in Table V-a. Clinical examination was carried out on the lines suggested in the circular of the Board of Education, England (1937).

TABLE V.

Average height and weight of children in Bijapur district.

Age (years).	Number of boys.	Number of girls.	AVERAGE HEIGHT (INCHES).		AVERAGE WEIGHT (LB.).	
			Boys.	Girls.	Boys.	Girls.
5	36	29	37.7	39.5	30.2	32.3
6	217	170	41.1	41.5	37.2	36.8
7	226	251	42.4	42.5	39.9	40.1
8	316	246	44.8	44.6	43.70	42.7
9	231	160	46.1	46.1	46.9	45.2
10	303	127	48.05	48.07	50.8	50.7
11	203	54	50.6	50.0	54.04	54.8
12	243	94	53.2	51.7	59.9	59.8
13	203	36	54.2	53.5	64.1	64.6
14	150	...	56.6	...	71.6	...

TABLE V-a.

*Results of a clinical examination of 5,131 children
in Bijapur district during the end of the
famine year 1943.*

Excellent, per cent.	Good, per cent.	Slightly sub-normal, per cent.	Bad, per cent.
0.21	37.92	53.22	8.64

SUMMARY.

1. A dietary survey of 42 families in different income groups was carried out during the end of famine year 1943.

2. The average calorie intake was sufficient in all income groups but some 33 per cent of families in the lower income groups consumed below 2,600 calories. Diets were deficient as regards animal protein, animal fat and calcium in all groups, vitamins A and C intake was also deficient in a number of families.

3. The quality of diet showed improvement with the increase in income.

4. Children belonging to different income groups, 5,131 in number, were examined for deficiency diseases and state of nutrition. The effects of famine conditions were reflected in a lowered state of nutrition of children, in an abnormally high prevalence of vitamin A deficiency, and to a lesser extent in the prevalence of vitamin C deficiency. There was also an increased death rate among infants and children between 1 and 10.

ACKNOWLEDGMENTS.

My thanks are due to the District Health Staff for their help in carrying out the survey and particularly to Dr. Konnur and Mr. Yadwad, the Sanitary Inspector, for their keen interest in carrying out the dietary inquiry in the families.

I am also grateful to Dr. K. A. Gandhi, D.P.H., D.T.M. & H. (Lond.), etc., Director of Public Health for the Government of Bombay, for allowing me to publish this paper and for giving advice and valuable guidance, and to Dr. W. R. Aykroyd, Director, Nutrition Research Laboratories, Coonoor, who encouraged me to prepare this paper.

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BLOOD UREA CLEARANCE IN INDIANS.*

BY

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KIDNEY function tests are based on a measurement of the capacity of the kidney to clear waste products of metabolism, such as urea, creatine, etc., from the blood in a given time. Analysis of either blood alone or urine alone would give only one side of the picture. More complete information is obtained by comparing blood and urine composition simultaneously. Such tests are classified as 'Clearance Tests'. The advantages and disadvantages of such tests have been discussed in detail by Herrin (1941) and Beaumont and Dodds (1944). One such test widely used in the clinical laboratory is the 'Blood Urea Clearance Test' of Möller, McIntosh and van Slyke (1929). This test has been stated to yield results bearing fairly close correspondence with the clinical findings in patients. For the sake of comparison, the authors made 120 standard clearance determinations on 18 normal subjects and 158 maximum clearances on 20 normal subjects. The standard for normal clearance was based on these observations in American subjects. The average values given by them were 54 c.c. for standard clearance (Cs) and 75 c.c. for maximum clearance (Cm), for a mean body-surface area of 1.73 square metres. Since the publication of these results the standards suggested by these authors have been adopted for assessing the urea clearance in health and disease in other countries.

During the urea clearance investigations on patients, Gokhale (1941) working at the Haffkine Institute, Bombay, found that the values for urea clearance were very low as compared to the standards set by Möller *et al.* (*loc. cit.*), although there were no clinical symptoms of kidney dysfunction. On account of such discrepancies in correlating the urea clearance values obtained in Indian patients with the clinical findings, he undertook a study of urea clearance in normal Indians. He investigated 110 subjects in Bombay and found that the average urea clearance either maximum or standard in normal Indians was only 60 per cent of the American standard. According to his findings, the average standard clearance is 31.5 c.c., and the maximum clearance is 40.01 c.c. without correction for the body-surface area; and 33.8 c.c. and 44.0 c.c. respectively after correction for the body-surface area of 1.73 square metres.

This investigation was followed immediately by that of Srikantia and Shamanna (1944) in Mysore. They studied 57 Mysoreans and their figures for the average standard clearance were 35.95, and for the maximum clearance 46.83 c.c., without the correction for the body-surface area; and 39.36 c.c. and 52.26 c.c. respectively after the necessary correction assuming the body-surface area to be 1.73 square metres.

It is a routine procedure in a hospital to scrutinize the clinical data on patients from time to time. At the Tata Memorial Hospital, besides the routine clinical investigations, urea clearance test is carried out in patients, for assessing their kidney function, before they are submitted to any major operative procedure. During the analysis of the data on urea clearance, it was found that here also the values were far below the standards of Möller *et al.* (*loc. cit.*) although no clinical symptoms of kidney dysfunction were observed in the patients. In the light of the findings of Gokhale (*loc. cit.*) and of Srikantia and Shamanna (*loc. cit.*), it was, therefore, considered worth while to report the data from this hospital.

METHOD.

The technique followed for the blood urea clearance test was the same as that described by Möller *et al.* (*loc. cit.*). The non-protein nitrogen content of serum and urea nitrogen

* This study was carried out under the direction of Dr. V. R. Khanolkar.

of blood were estimated by the method of nesslerization (Folin, 1934). The value for blood urea was obtained by multiplying the value for the urea nitrogen by the factor 2.14. The urinary urea was directly determined by the hypobromite method (Hawk and Bergeim, 1938). The results are shown in Table I:—

TABLE I.
Serum non-protein nitrogen and blood urea clearance in patients.
(Standard urea clearance.)

Serial No.	Age.	Sex.	Non-protein nitrogen, mg., per cent.	Blood urea clearance, c.c.	Nature of the pathological condition.
1	60	M	28.1	45.13	Benign hypertrophy of prostate.
2	65	F	19.5	27.00	Carcinoma corpus uterus.
3	60	F	18.0	39.40	„ vagina and vulva.
4	40	M	20.4	38.80	„ penis.
5	55	M	20.0	38.10	„ extrinsic larynx.
6	57	M	15.8	19.70	„ tongue.
7	45	M	22.5	46.80	„ mid. 1/3 œsophagus.
8	55	M	24.3	36.60*	„ stomach.
9	54	M	25.0	39.20	„ prostate.
10	46	M	20.0	31.80	„ ascending colon.
11	50	M	18.7	42.60	„ bladder.
12	58	M	21.4	30.90*	„ lower end œsophagus.
13	46	F	22.5	41.60	Multiple polyposis of rectum and colon.
14	46	M	23.7	19.70*	Healed chronic duodenal ulcer.
15	70	F	18.0	24.30	Carcinoma right breast.
16	62	F	21.4	16.60†	„ bladder.
17	57	F	19.6	33.20	Chronic inflammatory mass right breast.
18	30	F	30.0	27.70†	T. B. cervix.
19	45	F	17.1	39.40	Carcinoma lower end œsophagus.
20	53	M	23.7	48.00	„ stomach.
21	60	M	19.6	31.80	„ „
22	55	F	32.1	30.20	„ left breast.
23	52	M	23.7	42.60	„ stomach.

* The patients who expired after operation.

† Urea concentration was done in these patients and it was within normal limits.

TABLE I—*concl'd.*

Serial No.	Age.	Sex.	Non-protein nitrogen, mg., per cent.	Blood urea clearance, c.c.	Nature of the pathological condition.
24	50	M	19.6	49.80	Carcinoma transverse colon.
25	46	M	30.0	49.00	„ stomach pyloric end.
26	50	M	23.0	28.90	„ urinary bladder.
27	52	F	24.3	22.00*	„ right breast.
28	47	M	21.4	27.50	Leukæmia.
29	45	M	25.0	51.30	Carcinoma rectum.
Mean ...	52	...	22.4	35.16	
Maximum	70	...	32.1	51.30	
Minimum ...	30	...	15.8	16.60	
Maximum clearance.					
1	44	M	22.5	40.00	Healed chronic duodenal ulcer.
2	54	M	20.9	33.10	Carcinoma stomach.
3	67	M	22.5	56.80	„ intrinsic larynx.

* Urea concentration was done in these patients and it was within normal limits.

DISCUSSION.

The total number of cases is 32 out of which 29 had standard clearance and 3 had maximum clearance. These patients were admitted to the hospital with a probable diagnosis of a malignant disease in one of the internal viscera. The final diagnosis is given in the last column of Table I. None of these 32 patients had a kidney tumour. Their ages ranged from 30 to 70 years, the average being 52 years. These patients did not belong to any particular province but came from different parts of India. They were accustomed to a mixed diet, with smaller amount of meat as compared to the Europeans.

Blood urea clearance.

The values of standard clearance as given in Table I range from 16.60 c.c. to 51.30 c.c. with an average of 35.16 c.c. There were only three cases of maximum clearance, so no attempt has been made to find out the mean of their values. The figures for urea clearance when evaluated on the basis of standards given by Möller *et al.* (*loc. cit.*) suggest an impairment of renal function in the majority of the cases. There were no extra-renal causes such as vomiting, diarrhoea, etc., that would have affected the values of urea clearance in these patients. The non-protein nitrogen content of serum in these cases was also within normal limits. They did not show any other clinical symptoms of kidney dysfunction. All the patients made uneventful recoveries after major operative procedures, with the exception of three cases who expired after operation. It was, therefore, difficult to correlate these figures with the clinical findings.

One other obvious reason for low urea clearance might have been the age of these patients. The majority of the patients were between 45 and 60 years. Even after the application of an age correction factor, $\overline{UC} = 136.6 - 0.912 \times A$ where \overline{UC} = urea clearance in per cent of normal clearance value and A = age in years (Lewis and Alving, 1938), no correlation seemed to exist between the physical condition of the patients, the functional capacity of their kidneys, and the urea clearance values that were obtained. It was, however, found that if instead of evaluating the clearance on the basis of the American standard, the values suggested by Gokhale (*loc. cit.*) and by Srikantia and Shamanna (*loc. cit.*) were to be adopted, all these patients could be included in the category of persons within the range of normal urea clearance (Table II) :—

TABLE II.

Comparison of standard clearance values without the correction for the body-surface area.

			America.	Bombay.	Mysore.	Tata Memorial Hospital.
Mean	54.5	31.50	35.95	35.16
Maximum	68.3	43.62	51.64	51.30
Minimum	40.1	17.02	25.34	16.60

The findings of Gokhale (*loc. cit.*) and of Srikantia and Shamanna (*loc. cit.*) have recently been commented upon in the *Nature* (1945) and it is remarked that 'the figures of Gokhale and of Srikantia and Shamanna suggest that the Indian kidney has only about two-thirds the efficiency of its American counterpart; but whether this is true or not, it is obvious that a new and lower normal standard will have to be adopted for clinical work on Indians. Further, the authors suggest that the lower clearance of Indians, with their blood urea almost equal to that in Americans but the urinary urea less in the former than in the latter, is related to the lower protein content of their diet'.

We are in essential agreement with the opinion expressed above and would recommend the collection of further data from different parts of India to establish normal standards in Indians.

SUMMARY.

1. A study of non-protein nitrogen content of serum and of blood urea clearance test in 32 patients has been presented. Most of these patients were admitted for malignant disease, though none of them had a kidney tumour or any clinical evidence of kidney dysfunction.

2. The values for standard clearance ranged from 16.60 c.c. to 51.30 c.c. with an average of 35.16 c.c.

3. These values appeared to indicate an impaired kidney function on the basis of standards suggested by Möller *et al.* (*loc. cit.*) for Americans, but normal function on the basis of standards for Indians suggested by Gokhale (*loc. cit.*) and by Srikantia and Shamanna (*loc. cit.*).

4. The implications of these differences have been discussed and the need for collection of further data for normal standards in Indians has been advocated.

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OBSERVATIONS ON THE PHYSIOLOGICALLY ACTIVE FRACTION OF INDIAN HEMP, *CANNABIS SATIVA* LINN.

BY

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THOUGH Indian hemp is no longer officially recognized in the British or the United States Pharmacopœias, possibly because of the wide variations in potency exhibited by various brands of the substance either in the fresh state or after storage, interest continues to centre around this drug in India. It is not only used widely as an intoxicant all over India but medicinal preparations, either in the form of an extract or a tincture of hemp for internal or external use, are still in fair demand in hospitals and pharmacies.

Earlier workers reported that the active ingredient of Indian hemp was not an alkaloid (Smith, 1846) but a resin (Wood, Spivey and Easterfield, 1896, 1899) with a boiling point of 265°C. and a chemical composition of $C_{18}H_{24}O_2$ ('crude cannabinal' of Cahn, 1930). Later workers (Cahn, 1933; Myttenære, 1938, 1940), however, thought that cannabis resin contained more than one constituent, one of which might be cannabinal, $C_{21}H_{36}O_2$, $\left[\alpha \right]_D^{20} = -108^\circ$.

Interest in the chemistry of hemp resin has been recently revived by Work, Bergel and Todd (1939), Jacob and Todd (1940), Haagen-Smit *et al.* (1940), Adams, Hunt and Clark (1940), and Todd (1940). The present position of the knowledge in this field has been admirably reviewed by Todd (1943).

The estimation of the narcotic potency of hemp drug is important, particularly from the point of view of excise administration in India where the drug is permitted to be sold under Excise Department control. As the biological test suffers from certain limitations, several chemical and physical tests such as Beam's test, Vanillin test, Polarimetric test, etc. (Bose and Mukerji, 1943) were devised to measure this activity. None of these has, however, been found to be of any real value in measuring the potency of the hemp resins.

A new colorimetric test has been suggested by Mukhopadhyaya *et al.* (1943). The test is based on the hypothesis that all the pharmacologically active constituents of hemp resin are alkali-soluble phenols and that cannabinal, one of the important members of this group, is insoluble in dilute alkalis. Accordingly, the CCl_4 extract of charas is treated with 0.5 per cent NaOH solution, and an aliquot part is coupled with diazo-para-nitraniline resulting in the formation of a solid dye. This is matched against the colour produced by a standard sample of charas which has been similarly treated. The intensity of colour is claimed to run parallel with the change in the physiological potency of hemp as evidenced by the opinion of habitual smokers. In order to confirm these chemical findings, systematic pharmacological tests were undertaken in this Laboratory. The findings recorded in this paper represent experiences gained with samples either supplied by the Customs Control Laboratories or prepared in this Laboratory from samples secured from authentic sources.

EXPERIMENTAL.

1. *Method of extraction.*—One gramme of charas was exhaustively extracted in a Soxhlet extractor with 75 c.c. of carbon tetrachloride. The extract was shaken with four consecutive portions of 20 c.c. of 0.5 per cent NaOH solution. The alkali extracts were mixed together

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and the volume made up to 100 c.c. with distilled water. This was acidified and extracted with petroleum-ether. The residue in every case was taken up with CCl_4 .

In this way the following fractions were obtained:—

Fraction A = CCl_4 extract of charas.

Fraction B = CCl_4 extract of the alkali-insoluble portion of charas.

Fraction C = CCl_4 extract of the alkali-soluble portion of charas.

Fraction D = Petrol-ether extract of the alkali-soluble portion of charas.

Fraction E = CCl_4 solution of the alkali-soluble but petrol-insoluble fraction of charas.

A comparative biological evaluation of the narcotic properties of these fractions is given below.

2. *Laboratory details.*—A modified form of Gayer's (1928) test was employed on cats for the study of the narcotic properties of hemp resins. Animals within a certain range of weight

TABLE I.

Action of CCl_4 or petroleum-ether extract of various fractions of charas resin in cat.

Experiment No.	FRACTIONS : A.	B.	C.	D.	E.
	Minimum effective dose, c.c./kg.	Minimum effective dose, c.c./kg.	Tolerated or ineffective dose, c.c./kg.	Tolerated or ineffective dose, c.c./kg.	Tolerated or ineffective dose, c.c./kg.
1	0.5	0.2	1.0	1.0	...
2	1.5	0.5	1.0	2.0	...
3	1.0	0.7	3.0	3.0	...
4	1.0	0.6	1.5	2.0	...
5	1.5	1.0	2.5	2.5	...
6	2.5	1.5	3.0	2.5	...
7	3.0	2.0	5.0	5.0	...
8	0.7	0.5	2.0	2.5	...
9	2.0	1.0	5.0	4.0	...
10	0.6	0.4	2.0	2.0	...
11	2.0	1.0	...	3.0	3.0
12	2.0	0.5	...	3.0	3.0
13	2.0	1.0	...	3.0	3.0
14	1.5	0.7	...	3.5	3.5
15	2.0	1.5	...	4.0	4.0
16	1.0	0.6	...	5.0	5.0
Mean	...	1.6	0.3	2.6	3.6

(2 kg. to 3 kg.) were kept under laboratory conditions for 4 days prior to the starting of the experiments and they were fasted overnight before the drug was administered in the morning. 'Sensitive' cats were picked out by preliminary trial treatments of a dosage of solid extract of *Cannabis sativa* or by the CCl_4 extract of whole charas. The drugs were given in a small volume of 0.5 c.c. to 0.6 c.c. of alcohol which *per se* did not have any effect on the animal. In case of CCl_4 extracts, the solvent was evaporated off *in vacuo* because of the possible toxic effect of carbon tetrachloride, and the residue was taken up with alcohol and given through a Ryle's stomach-tube. The minimum effective dose of whole charas was then worked out from every positive animal and comparative effects of varying doses of other fractions were similarly estimated on the same animal on every third day. The maintenance of the initial level of sensitiveness in every animal was checked from time to time by the interpolation of the initial effective-dose of whole charas. During experimentation, animals were kept in seclusion in a semi-dark room with a view to minimize all external stimuli capable of diminishing the degree of responses. The animals were observed for a period of 4 to 5 hours from the time of administration of the drug and a positive response could usually be elicited within 1½ to 3 hours after the drug was given. Though over 50 cats were used for this study, only 16 cats were found to be 'sensitive' to the action of charas. Positive cannabis response was indicated by a brief period of excitement followed by dragging of hind limbs, inability to walk straight, tendency for circular movements, ataxia, rocking movements of the head, somnolence and even mild narcosis. Though sluggish corneal reflex was elicited in most of the animals, definite corneal anaesthesia as reported by Gayer (*loc. cit.*) was not observed in the test animals. The results are shown in Table I.

It will be observed that the 'total extract' (fraction A) and the 'alkali-insoluble extract' (fraction B) of charas showed strong narcotic properties. The activity observed in the CCl_4 extract of the drug proves that the active principles of hemp are soluble in it. The greater potency of the alkali-insoluble fraction indicates that some of the inactive constituents are eliminated by the alkali treatment of charas. Complete absence of all narcotic effects in 3 to 4 times higher dosages of the petrol-ether or carbon-tetrachloride extracts of the alkali-soluble fraction proves beyond all doubt that the active principle or principles are not soluble in dilute NaOH solution.

Our results, therefore, do not corroborate the findings recorded by Mukhopadhyaya *et al.* (*loc. cit.*) that the narcotic power of hemp drugs could be estimated by a colorimetric reaction. The alkali-soluble fraction, on which the 'Mukhopadhyaya test' is based, is apparently devoid of all biological activities. The alkali-insoluble fraction represents the pharmacologically active portion of the hemp resin. From what is already recorded in the published literature concerning the differential solubilities in alkali of the various resinoid fractions of hemp, it may be presumed that the CCl_4 solution of alkali-insoluble fraction should contain cannabinol and some of its isomers.

3. *Action of homotetrahydrocannabinol.*—Bose and Mukerji (*loc. cit.*) suggested on theoretical considerations that possibly 'cannabinol' might be the precursor of the true active ingredient in hemp, the phenol being broken down in the system to some other derivative possessing the ethenoid linkage but with more intense physiological activity. The synthesis by Adams *et al.* (*loc. cit.*) of some tetrahydrocannabinols possessing strong hemp activity appears to lend support to this supposition. In order to correlate, if possible, the action of one such compound with the action obtained with the alkali-insoluble fraction of charas, a sample of synthetic homotetrahydrocannabinol (i.e., the analogue of tetrahydrocannabinol containing an n-hexyl chain) was procured from Prof. Todd of Manchester and its narcotic action carefully studied on cats and rabbits and compared with the above results. Homotetrahydrocannabinol was found to be insoluble in water and in dilute alkalis. It is soluble in alcohol and in carbon tetrachloride. An alcoholic solution was, therefore, made and the doses given in a small volume of 0.5 c.c. to 0.6 c.c. intraperitoneally into every animal. For economy of substance is inevitable. The sensitivity of the animals was measured against a solid extract of cannabis, given orally in 0.5 c.c. to 0.6 c.c. of alcohol. The comparative results are shown in Table II.

TABLE II.

Effect of homotetrahydrocannabinol compared to the solid extract of Cannabis sativa in cat and rabbit.

Experiment No. (Series).	CAT.		RABBIT.	
	SOLID CANNABIS EXTRACT.	HOMOTETRAHYDRO- CANNABINOL.	SOLID CANNABIS EXTRACT.	HOMOTETRAHYDRO- CANNABINOL.
	Positive response dose, mg./kg.			
1	100	15	120	20
2	80	15	100	20
3	70	10	130	20
4	80	15	110	25
5	90	20	100	25
Mean	...	84	112	22

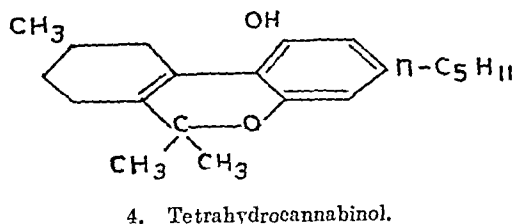
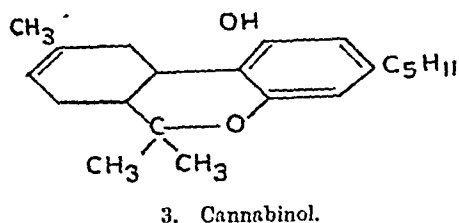
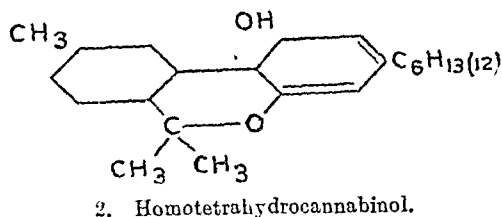
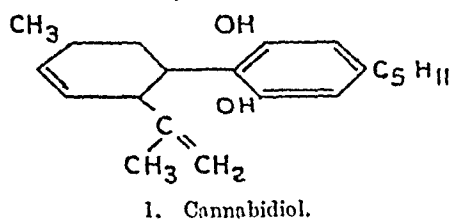
Homotetrahydrocannabinol is therefore physiologically active. Dose for dose, its activity is greater than that of the solid extract. The nature of action was found to be very similar to that obtained with the CCl_4 extract or the alkali-insoluble fraction of the hemp resin and the same types of inco-ordination, ataxia and narcotic manifestations were observed. The ataxic dose of homotetrahydrocannabinol is high in both the species of animals but cats are more suitable and more sensitive animals than rabbits for the study of the ataxia manifestations.

DISCUSSION.

A study of the residues obtained from the different fractions of charas indicates that the solid matter contained in the minimum effective dose of the active fraction of charas (8 mg./c.c. to 10 mg./c.c.) is quantitatively smaller than the active dose of homotetrahydrocannabinol. The residue is a grossly impure material contaminated by inert substances like terpene, hydrocarbon, cannabinol, cannabidiol, etc. The active principle should, therefore, represent only a minor fraction of this total solid. Judging from this, it appears that the levorotatory natural principle of charas is likely to be very much more potent than the synthetic homotetrahydrocannabinol which is optically inactive, and which is probably the most active of all constituents so far isolated from the natural hemp resin.

Todd (*loc. cit.*) has discussed in detail the probable biogenesis of natural and synthetic compounds possessing 'hashish' (hemp) activity. Though tetrahydrocannabinols have not yet been successfully isolated from natural sources, it appears that cannabidiol, tetrahydrocannabinol and cannabinol all occur in nature from the condensation of terpene derivatives with olivetol. The first condensation product is cannabidiol which is physiologically inactive.

From this, by a process of cyclization, the active tetrahydrocannabinol is formed. This in turn by dehydrogenation becomes the inactive cannabiniol. The structural relationship of these compounds, according to Todd, is as follows:—



It is very likely that the hemp resins contain a mixture of active isomeric tetrahydrocannabinols. Homotetrahydrocannabinol is possibly one of them. The similarity of action of this with that obtained with the alkali-insoluble fraction of charas lends support to the hypothesis that one such compound may be present in natural charas for producing its narcotic action. It is interesting to note that, as early as 1846, Smith observed 'hashish' activity in the alkali-insoluble portion of the resin, though this work has been hardly noticed in the published literature of comparatively recent times.

SUMMARY AND CONCLUSIONS.

1. A comparative pharmacological study of the alkali-soluble and alkali-insoluble fractions of charas in CCl_4 and petrol-ether solvents has been made. The total extract and the alkali-insoluble fraction were found to possess the narcotic properties of the drug as evidenced by the production of ataxia in cats. The alkali-soluble fractions were found to be devoid of all physiological activities.

2. Most of the chemical and colorimetric tests including the test recently described by Mukhopadhyaya *et al.* (*loc. cit.*) depending on the presence of phenols in the alkali-soluble portion of the CCl_4 extract, do not measure the narcotic potency of hemp drugs.

3. A comparison of the action of homotetrahydrocannabinol and the alkali-insoluble fraction of charas shows that the former or an allied compound is possibly the active principle for the narcotic effect of hemp.

4. The activity of the alkali-insoluble portion of hemp resin is greater than that of homotetrahydrocannabinol. This suggests that the alkali-insoluble portion probably contains some optical isomers of tetrahydrocannabinol which are more active than tetrahydrocannabinol or its optically inactive homologues.

ACKNOWLEDGMENT.

We wish to thank Prof. A. R. Todd, F.R.S., formerly Director of the Chemical Laboratories, University of Manchester, for his courtesy in forwarding to one of the authors (B. M.) a sample of homotetrahydrocannabinol synthesized by him.

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TABLE II.

Effect of homotetrahydrocannabinol compared to the solid extract of Cannabis sativa in cat and rabbit.

Experiment No. (Series).	CAT.		RABBIT.	
	SOLID CANNABIS EXTRACT.	HOMOTETRAHYDRO- CANNABINOL.	SOLID CANNABIS EXTRACT.	HOMOTETRAHYDR CANNABINOL.
Positive response dose, mg./kg.				
1	100	15	120	20
2	80	15	100	20
3	70	10	130	20
4	80	15	110	25
5	90	20	100	25
Mean	...	84	112	22

Homotetrahydrocannabinol is therefore physiologically active. Dose for dose, its activity is greater than that of the solid extract. The nature of action was found to be very similar to that obtained with the CCl_4 extract or the alkali-insoluble fraction of the hemp resin and the same types of inco-ordination, ataxia and narcotic manifestations were observed. The ataxic dose of homotetrahydrocannabinol is high in both the species of animals but cats are more suitable and more sensitive animals than rabbits for the study of the ataxia manifestations.

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ANÆMIA IN INDIAN 'ARMY RECRUITS.

BY

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INTRODUCTION.

HITHERTO most studies of anæmia in India have been concerned with the severe macrocytic anæmia (Nutritional Macrocytic Anæmia—N.M.A.) first described by Balfour (1927) and Lucy Wills (1931, 1934). and though milder anæmias are thought to be common in the whole population, little has been done to determine their incidence and nature.

Macdonald (1939) estimated the hæmoglobin of several thousand coolies on tea-estates in Assam, and found that even the men were invariably anæmic—none had over 13 g. hæmoglobin per 100 ml., and 90 per cent had less than 11 g. Hare (1940) found that new recruits to the estates were as anæmic as old inhabitants, and it has been concluded that anæmia is probably universal amongst poorer Indians. Little is known of the nature of this common anæmia. Napier and his school (*see* Napier and Das Gupta, 1942) did hæmatocrit determinations on a relatively small number of Assam tea-garden coolies, and found that the anæmia was microcytic but normochromic. It was not cured by iron. These milder anæmias have not seemed to be related to hookworm infestation. Since no other cause has been established they are regarded simply as a consequence of the malnutrition so common in India. The relatively high iron content of the common Indian diet has been thought to preclude iron deficiency as a major cause of the anæmia, and in the few trials reported iron medication did not cure it.

The present authors (Hynes *et al.*, 1945) found that of North West Indian soldiers in Peshawar district only one-third had over 14 g. hæmoglobin per 100 ml., and nearly all the anæmia present was hypochromic and easily curable by iron. This finding was so at variance with the earlier reports mentioned above that we decided to make a specific search for iron deficient anæmia in recruits.

The blood status of the general population cannot be accurately determined by a blood survey of army recruits, for they are a selected sample of the population from which the recruiting medical officer has excluded all obviously diseased members. Nevertheless, even if by examining recruits we underestimate the amount of anæmia in the population, we may still fairly assume that we are drawing a true sample of the types of anæmia present.

Of recruits drawn from the labouring classes 25 to 40 per cent were anæmic, and this is probably an underestimate of the amount of anæmia present in unselected men of this class. It must be even commoner in women, who have to face the blood demands of child-bearing and menstruation.

MATERIAL AND METHODS.

Material.

We determined the hæmoglobin, red cell count, and packed cell volume of some 600 newly joined recruits in the I.A.M.C. Training Centre, Rawalpindi, between June and November 1944. The number of each race and class examined can be seen in Table I. The majority were sweepers and ward servants, a miscellany drawn from the poorer and non-martial classes. Ambulance sepoy's are drawn from the same class as infantry recruits, but

are probably of a somewhat lower physical standard. Nursing sepoy come from a more prosperous class than any of the above, for the army demands that they shall have reached a relatively high educational standard (Standard IX).

Technique.

Blood.—Blood was drawn from an arm vein with the minimum of stasis. Syringes were sterilized by hot liquid paraffin, and Wintrobe's dry oxalate mixture was used as anti-coagulant (Whitby and Britton, 1942). All blood was taken between 08.00 and 09.00 hours.

Hæmoglobinometer.—Hæmoglobin was estimated as acid hæmatin matched against a glass wedge standard in the Zeiss 'Hæmometer'. We re-calibrated the instrument in January 1945 with bloods of known hæmoglobin content determined as alkaline hæmatin with a standard made from pure crystalline hæmin, B.D.H. (King *et al.*, 1944). The standard error between duplicate readings with this instrument is ± 0.227 g. Thus, a difference between two readings exceeding 0.5 g. is statistically significant ($P=0.03$), and the difference should exceed 0.6 g. in only 0.8 per cent of trials, and 0.7 g. in 1 trial in 500. A difference of 0.6 g. was in fact found in 4 of 635 duplicate readings; there was no larger difference. The standard error of a single reading is ± 0.16 g.

Red cell counts.—We used Adams and Zeiss red cell counting pipettes. Two Zeiss Thoma counting chambers were charged from the same pipette and about 500 cells counted in each. If the totals for the two chambers differed by more than 10 per cent another pipette was filled and the count was repeated.

Hæmatocrits.—The packed cell determinations were done in Wintrobe hæmatocrits; 45 minutes' spinning at 3,000 r.p.m.

Hookworm examinations.—We examined stools for hookworm ova by the usual gravity-flotation technique in which about 1 g. of faeces is emulsified in 10 ml. of saturated saline. We counted the number of ova per microscope field (1/3" objective and $\times 6$ ocular) and roughly classified the infestation as:—

Very light:—1 ovum per 6 or more fields.

Light:—1 ovum per 2 to 5 fields.

Moderate:—1 to 4 ova per field.

Heavy:—5 or more ova per field.

Statistics.—The statistical methods used are those of Fisher (1944). The significance of differences between means was tested by the 't' test, and between proportions by the χ^2 test. When the latter reduced to a fourfold table the method of Fisher and Yates (1943), Table VIII, was used. In the text we give only the probability (P) that the difference was due to chance; e.g. ($P=0.01$) means that the difference in question might be due to chance only once in 100 trials.

THE AMOUNT OF ANÆMIA PRESENT.

The hæmoglobin distribution in the various groups is summarized in Table I and Graph 1.

The most striking feature is the great superiority of the nursing sepoy over other recruits of the same race; in all four cases the consequent difference in mean hæmoglobins was statistically significant. Only 8 per cent of the nursing sepoy recruits were anæmic (hæmoglobin less than 13 g.) and 80 per cent had over 14 g. hæmoglobin. This comparatively very low incidence of anæmia is probably explained by the higher social status and resultant better nutrition of these recruits.

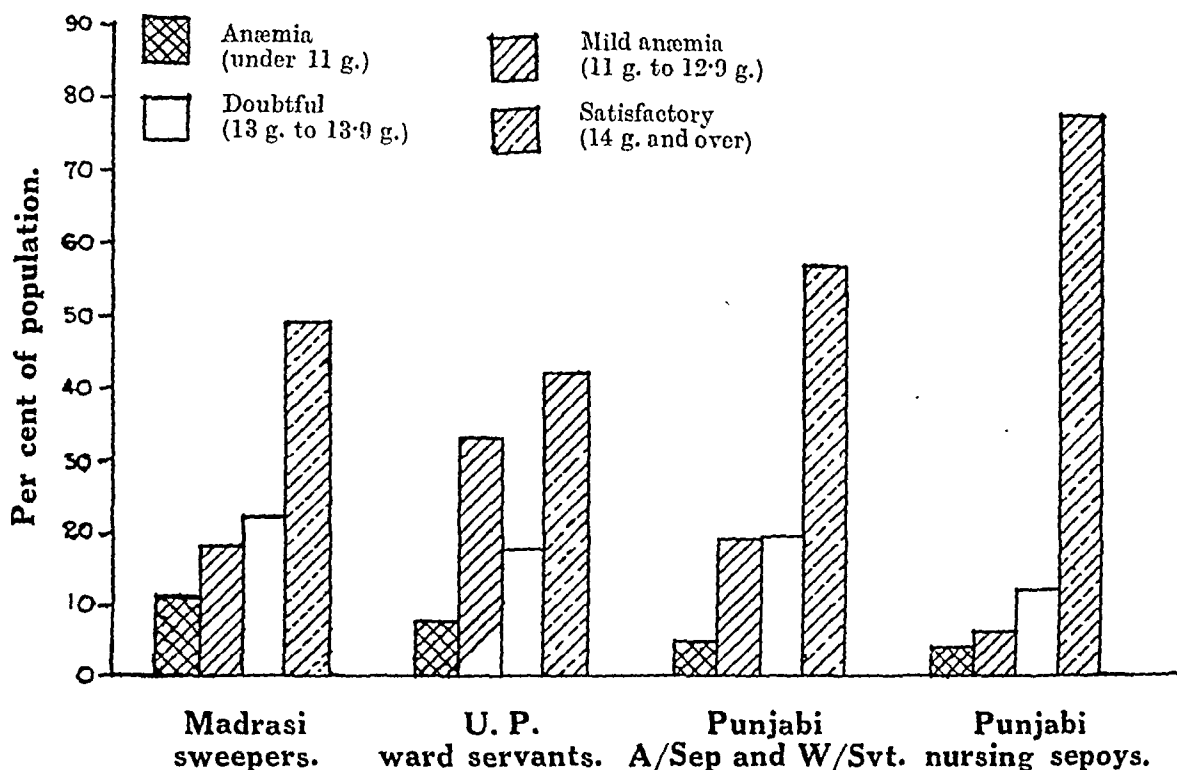
About 25 per cent of the Punjabi ambulance sepoy and ward servant recruits were anæmic (hæmoglobin less than 13 g.) and 5 per cent had less than 11 g. hæmoglobin. There was no significant difference between the two.

TABLE I.
The haemoglobin distribution of recruits from different provinces.

HEMOGLOBIN GRAMME PER 100 ML.																	
Province.	Class.													Total.	Mean.	S. D.	
		6-	7-	8-	9-	10-	11-	12-	13-	14-	15-	16-	17-				18-
Madras	Sw'pr	3	4	14	13	22	42	43	36	13	2	...	192	13.66	1.831
U. P.	W/Svt	...	2	1	1	3	13	19	17	21	13	6	1	...	97	13.35	1.901
Punjab	A/Sep	5	7	13	13	25	14	6	83	13.83	1.595
	W/Svt	2	...	2	9	18	25	18	3	77	14.05	1.278
Kashmir	N/Sep	2	1	2	6	7	13	14	4	1	50	15.12	1.690
	W/Svt	1	2	5	7	4	19	13.97	1.200
C. P.	N/Sep	3	4	6	2	1	...	16	15.09	1.183
	W/Svt	1	2	...	3	4	1	11	13.15	2.484
N.-W. F. P.	N/Sep	1	4	4	1	...	10	16.03	0.709
	W/Svt	1	2	1	2	6	13.72	1.835
Bombay	N/Sep	2	4	2	8	15.35	0.859
	W/Svt	3	2	2	2	6	1	...	16	15.01	1.656
Sind	W/Svt	1	2	3	1	1	8	13.89	2.020
Calcutta	W/Svt	2	3	2	1	8	13.79	1.056
Sw'pr—Sweeper.		W/Svt—Ward servant.												A/Sep—Ambulance scopy.		N/Sep—Nursing scopy.	

About 40 per cent of both U. P.* ward servants and Madrasi sweepers were anæmic (hæmoglobin less than 13 g.), and the inferiority of the U. P. ward servants to the Punjabi ward servants was statistically significant ($P=0.02$). This difference may correspond to the known superiority of general nutrition in the Punjab to that in other parts of India.

GRAPH 1.



Showing the amount of anæmia in different categories of recruits.

The religion of an Indian influences both his social status and his dietary habits. The only significant difference in this series between religions or castes was that in both Punjabi and U. P. recruits, the mean hæmoglobin was significantly lower in Mohammedans than in Hindus ($P=0.02$ in both cases). These differences were probably economic in origin, for the Mohammedan has fewer dietary prejudices than the Hindu.

The majority of the recruits gave their ages as between 18 and 21, few were under 18 or over 35. There was no correlation between age and anæmia.

Hookworm infestation and anæmia.

Table II shows the degree of hookworm infestation in the five largest categories of recruits. About 60 per cent of both Madrasi and U. P. recruits were infested; the greater amount of moderately heavy infestation in the latter was statistically just significant ($P=0.04$). The Punjabis showed only half as much infestation, statistically a highly significant difference. Thirty per cent of Punjabi ambulance sepoy and nursing sepoy were infested, and there was no significant difference between them, but the Punjabi ward servants with only 12 per cent of infestation differed very significantly (P less than 0.005).

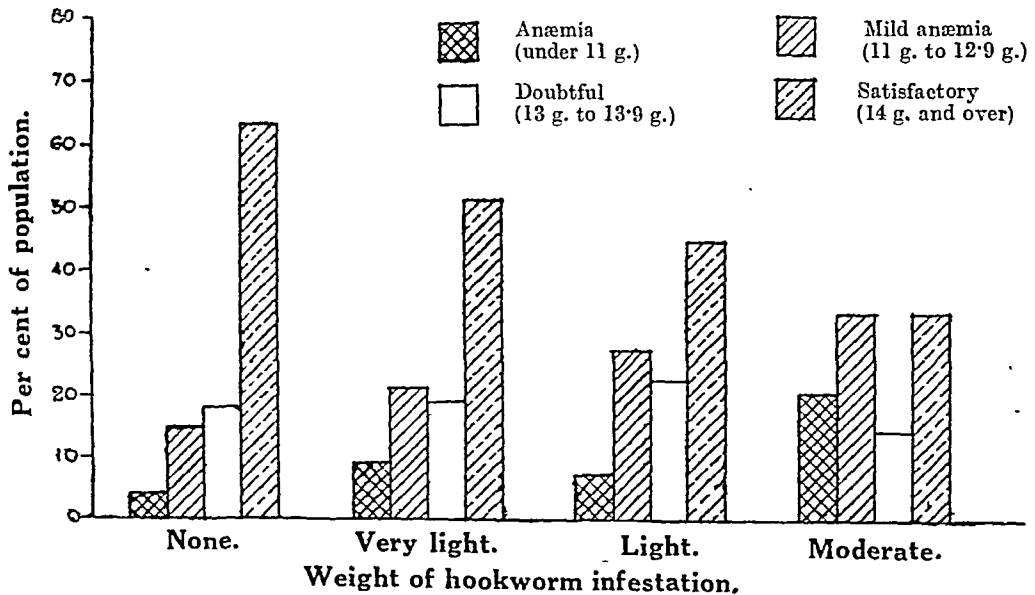
* United Provinces.

TABLE II.

The percentage of hookworm infestation in various classes of recruits.

Province.	Class.	Number of cases.	PERCENTAGE.			
			None.	Very light.	Light.	Moderate.
Madras	... Sw'pr	192	41	35	14	10
U. P.	... W/Svt	97	39	31	8	22
Punjab	... { A/Sep	83	71	20	5	4
	... { W/Svt	77	88	8	3	1
	... { N/Sep	50	68	14	8	10

GRAPH 2.



Comparing the amount of anæmia in recruits with different degrees of hookworm infestation.

We found a marked relation between hookworm infestation and anæmia. Table III shows the hæmoglobin distribution in recruits with the different degrees of infestation. The amount of anæmia increased progressively as the weight of the infestation increased, and the mean hæmoglobin fell from 14.3 g. in men with no infestation to 12.6 g. in men with a moderately heavy infestation. Even the men with only a very light infestation had a mean hæmoglobin significantly lower than that of non-infested men ($P=0.007$) and the difference between moderately and very lightly infested men was very highly significant (P less than 0.001). Graph 2 shows how the amount of anæmia increased as the infestation grew heavier.

On the other hand the connection between anæmia and hookworm infestation was by no means constant—one-third of recruits with under 11 g. hæmoglobin had no infestation, and one-third of the men with a moderately heavy infestation had over 14 g. hæmoglobin (Tables III and IV):—

TABLE III.

The hæmoglobin distribution in recruits with various degrees of hookworm infestation.

Weight of infestation.	HÆMOGLOBIN GRAMME PER 100 ML.												Total. Mean. S. D.		
	7-	8-	9-	10-	11-	12-	13-	14-	15-	16-	17-	18-			
None	3	2	6	11	36	58	82	68	39	9	1	315	14.31	1.629
Very light	1	1	11	11	18	26	26	30	15	1	...	140	13.82	1.823
Light	3	6	6	10	12	7	1	45	13.46	1.536
Moderate ...	2	...	4	4	8	8	7	10	5	1	49	12.63	2.049

TABLE IV.

The amount of hookworm infestation in recruits with different hæmoglobin levels.

Hæmoglobin class.	HOOKWORM LOAD, PER CENT.				Number of cases.
	None.	Very light.	Light.	Moderate.	
Under 11 g. ...	30	35	8	27	37
11 g. ...	45	28	12	15	104
13 g. ...	57	26	10	7	101
14 g. and over ...	65	23	7	5	307

We may conclude that hookworm infestation was a secondary factor in the anæmia of these recruits. In the presence of other more important causative factors it might increase the degree of anæmia, but even in substantial infestation normal hæmoglobin values were commonly seen.

THE TYPES OF ANÆMIA PRESENT.

Definitions.

The most satisfactory classification of anæmia is according to mean corpuscular volume (MCV) and hæmoglobin concentration (MCHC). Practically all recruits with hæmoglobin values over 14 g. per 100 ml. had MCV's between 80 cu.μ. and 99 cu.μ. and MCHC's of 30 per cent or over. These figures are the basis of the classification of anæmia used in this paper, viz. :—

MCV.

Microcytic : less than 80 cu.μ.
 Normocytic : 80 cu.μ to 99 cu.μ.
 Macrocytic : 100 cu.μ or over.

MCHC.

Hypochromic : less than 30 per cent.
 Normochromic : 30 per cent or over.

Findings.

The different types of anæmia found in each class of recruit are shown in Table V :—

TABLE V.

The number of cases of different types of anæmia (hæmoglobin under 13·0 g.) found in recruits.

Type of anæmia.		MADRAS.	U. P.	PUNJAB.		
		Sw'pr.	W/Svt.	A/Sep.	W/Svt.	N/Sep.
Microcytic	{ Hypochromic	7	...	2	1	1
	{ Normochromic	5	2	3	1	...
Normocyctic	{ Hypochromic	17	4	10	4	2
	{ Normochromic	25	31	10	7	2
Macrocytic	{ Hypochromic	1
	{ Normochromic	1	2
Total of anæmias ...		56	39	25	13	5

Normocyctic anæmia.—In all classes over three-quarters of the total anæmia was normocyctic. In both Madras and Punjab about 60 per cent of the normocyctic anæmia was normochromic, but in U. P. recruits 90 per cent was normochromic and only 10 per cent hypochromic. This difference was statistically highly significant (P less than 0·005).

Microcytic anæmia.—In both Madras and Punjab about 20 per cent of the total anæmia was microcytic, and about half the microcytic anæmias were normochromic. There was no significant difference between these two classes. In U. P. recruits only 2 of 39 cases of anæmia were microcytic, both were normochromic. This low incidence of microcytosis was a significant difference from the Madras ($P=0\cdot025$), but did not quite reach significance compared with the Punjab.

The proportion of microcytic to other anæmias was greater the more severe the anæmia, but no statistical significance attached to this.

Macrocytic anæmia.—Only 5 very mild cases of macrocytic anæmia were found—2 each from the Madras and the U. P., and 1 in a ward servant from Sind. One of the Madras had a hypochromic macrocytic anæmia; the remaining 4 were normochromic. None of these cases had bilirubinæmia or splenomegaly.

No case of macrocytic anæmia was found in the Punjab.

Hypochromia.—In both Madras and Punjab about 45 per cent of the anæmia was hypochromic, but in U. P. recruits the proportion was only 10 per cent, a highly significant difference (P less than 0·005).

The proportion of hypochromic anæmias increased as the anæmia became more severe, until with hæmoglobin values below 10 g., 11 out of 13 cases were hypochromic. χ^2 tests proved the significance of this observation. Extreme hypochromia (MCHC less than 28 per cent) was rarely seen except in the more severe anæmias—only 3 of 15 cases had over 11 g. hæmoglobin per 100 ml.

It might be anticipated that hypochromia, a sign of iron deficiency, would be more common in hookworm infestation, but in fact this was not the case. The proportion of

normochromic to hypochromic anæmia was the same in infested and non-infested men, even when the infestation was moderately heavy.

An MCHC of over 30 per cent must be classed as normochromic, but whereas 56 per cent of recruits with hæmoglobin over 14 g. had an MCHC of 32 per cent or over, this value was exceeded in only 35 per cent of the normochromic anæmias. This difference is statistically highly significant. Of course there is in fact no arbitrary MCHC which forms the boundary between normochromia and hypochromia; red cells with an MCHC below 30 per cent are certainly unsaturated, but those with a little over 30 per cent may be saturated or unsaturated.

THE EFFECT OF ARMY LIFE AND OF IRON SUPPLEMENTS ON THE ANÆMIA OF RECRUITS.

The object of the work described below was to observe to what extent the recruits' anæmia improved during training, and to determine whether the improvement could be accelerated by a small daily dose of iron. We, therefore, divided the recruits into a 'control group' and an 'iron group', both taking the same controlled diet, and gave 6 grains of ferrous sulphate daily to the iron group.

Material and methods.

Our subjects were 92 Madras sweepers and 74 U. P. ward servants taken at random, and 33 Punjabi ambulance sepoy, 23 Punjabi ward servants, and 35 miscellaneous ward servants with hæmoglobins less than 14 g., but otherwise taken at random. Up to 15 newly joined recruits were investigated each day, and alternate men as they presented themselves were placed in the iron and control groups.

TABLE VI.

The average daily diet of these recruits.

			Oz.	Prot., g.	Carb., g.	Fat, g.	Fe, mg.
Meat	4½	19	...	32	2·5
Milk	6	5·5	8	6	...
Ghee, animal	1½	36	...
Ata (wheat-flour)	20	65	390	9	40
Rice	4	10	95	1	3
Dhal (pulses)	4¼	21	84	4	10
Vegetables	4½	2	8	...	1·5
Potatoes	3¾	1·5	23	...	0·5
Onions	2	0·5	6·5	...	0·5
Fruit	1½	...	5
Ghee, vegetable	1	32	...
Sugar	2¼	...	63

Total calories : 4,200.

All the men were given the diet shown in Table VI. The diets were supervised by Major O. P. Verma, I.A.M.C., who was at the same time making observations on nutrition. For this reason about one-half of each group received an extra 1 lb. of milk daily; this had no demonstrable effect on the blood.

The iron group were given 6 grains of ferrous sulphate daily throughout the period of observation. The powder (ferrous sulphate exsiccatus B.P. manufactured by an Amritsar firm) was suspended in distilled water immediately before use. A single dose of 6 grains in $\frac{1}{2}$ oz. water was given daily at 08:00 hours. A member of the team always gave the medicine and watched that every man swallowed it. The men did not complain of alimentary or other disturbances.

The number of men in the experiment dwindled steadily owing to losses from sickness and training requirements. The loss was uniform between the iron and control groups, and between the different hæmoglobin classes.

Results.

Hæmoglobin.—The change to the army life and diet considerably improved the anæmia of these recruits, but the men taking daily iron did very much better. On recruitment both groups contained about 45 per cent of anæmic men (hæmoglobin less than 13 g.) and about 25 per cent of men with 14 g. hæmoglobin or over. After 3 months' training 31 per cent of the control group men were still anæmic, and 50 per cent had 14 g. or more, whereas in the iron group only 8 per cent of the men were anæmic and 80 per cent had 14 g. or more (Tables VII and VIII and Graph 3):—

TABLE VII.

The hæmoglobin distribution of the control group of recruits after 0, 1, 2, 3 and 5 months of training.

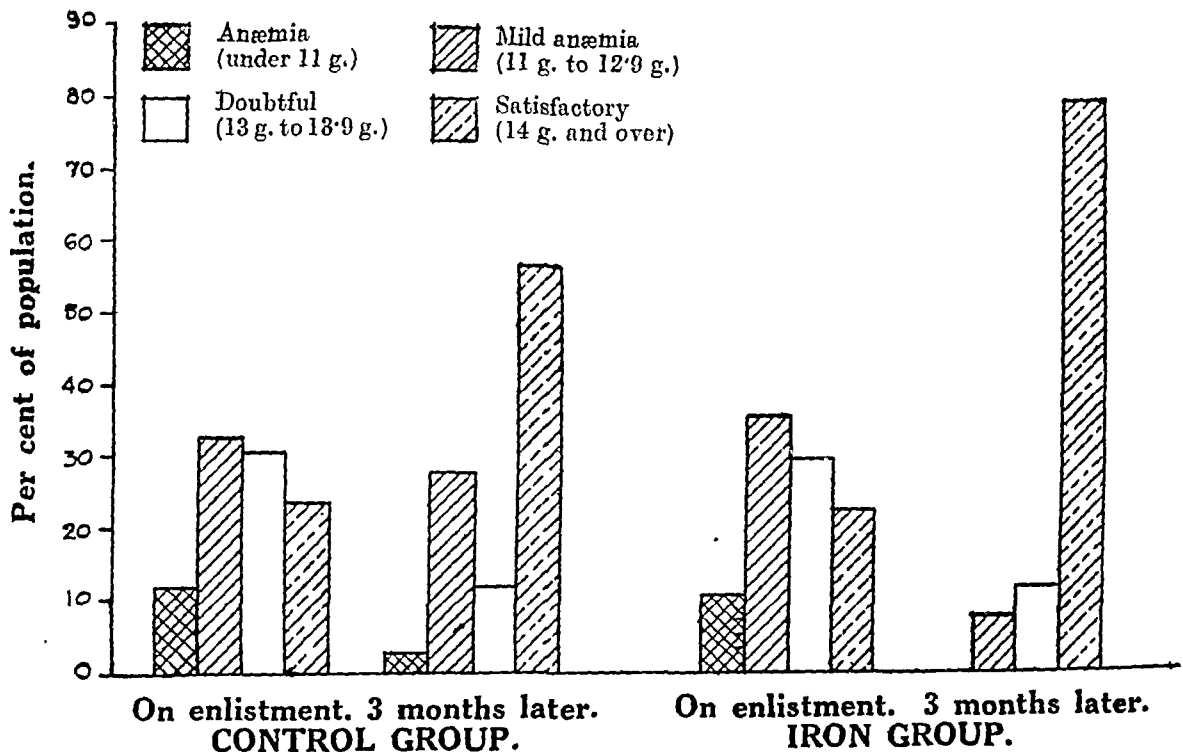
Month of service.	HÆMOGLOBIN GRAMMES PER 100 ML.											Number of cases.	Mean.	S. D.
	7-	8-	9-	10-	11-	12-	13-	14-	15-	16-	17-			
0	4	12	17	27	41	18	10	3	1	133	12.95	1.577
1	...	2	3	16	19	25	28	13	11	4	1	122	12.73	1.765
2	2	7	14	15	26	25	14	3	1	107	13.39	1.597
3	1	2	11	13	10	27	19	2	1	86	13.73	1.617
20 men	3	3	3	4	6	4	20	13.60	1.403
	5	1	2	3	7	4	3	20	13.40	1.322

TABLE VIII.

*The hæmoglobin distribution of the iron group of recruits after
0, 1, 2, 3 and 5 months of training.*

Month of service.	HÆMOGLOBIN GRAMMES PER 100 ML.												Num- ber of cases.	Mean. S. D.
	7-	8-	9-	10-	11-	12-	13-	14-	15-	16-	17-	18-		
0	2	3	2	7	17	27	37	18	9	2	124	12.88 1.672
1	...	1	2	2	15	26	36	26	10	2	120	13.30 1.395
2	2	3	7	22	40	19	6	2	...	101	14.22 1.264
3	7	10	22	29	12	2	1	83	14.83 1.242
18 men	3	1	1	9	6	1	18	14.74 0.978
	5	1	6	11	18	14.99 0.613

GRAPH 3.



Showing the amount of anæmia in recruits on enlistment and after three months' training the only difference between the two groups being that the 'iron group' received 6 grains ferrous sulphate daily.

On recruitment the two groups had almost identical mean hæmoglobin values, but after 1 month the iron group's mean hæmoglobin was significantly higher than the control

group's ($T=0.01$), and after 2 and 3 months the differences were very significant (P less than 0.001).

We were able to follow about 20 recruits from each group for 5 months. Those in the iron group (who all continued their iron) improved further, but there was no significant change in the control group. The hæmoglobin distributions of these men at both 3 and 5 months are shown in Tables VII and VIII.

Table IX shows the response in 3 months of the different grades of anæmia. The hæmoglobin rose significantly (0.6 g. or more) in 95 per cent of the anæmic men of the iron group and 66 per cent of those in the control group; the rise exceeded 1.0 g. in 86 and 53 per cent respectively. In the iron group the hæmoglobin rose significantly in 90 per cent of men with under 15 g. hæmoglobin, compared with 60 per cent in the control group.

TABLE IX.

The improvement after 3 months' training of men with different initial hæmoglobin levels.

Initial hæmoglobin.	Group.	Number of cases.	HEMOGLOBIN AFTER 3 MONTHS' TRAINING.					
			Under 11 g.	11 g.-	12 g.-	13 g.-	14 g.-	15 g. and over.
Under 11 g.	Iron	9	3	3	2	1
	Control	11	1	6	4
11 g.-	Iron	11	2	2	4	3
	Control	12	2	3	4	2	...	1
12 g.-	Iron	17	1	4	5	7
	Control	15	...	2	3	1	9	...
13 g.-	Iron	20	1	2	10	7
	Control	21	2	6	8	5
14 g.-	Iron	17	1	16
	Control	17	1	8	8
15 g. and over	Iron	10	10
	Control	10	2	8

Even the men with initial hæmoglobin values between 14 g. and 14.9 g. were capable of improvement—half of those in the control group and 16 of 17 in the iron group showed a significant rise in 3 months, and in 12 of the iron group this exceeded 1.0 g.

hand hookworm infestation was not essential to anæmia—one-third of men with less than 11 g. hæmoglobin had no infestation—nor was anæmia an invariable consequence of infestation—one-third of men with a moderately heavy infestation had over 14 g. hæmoglobin. This accords with the modern view that hookworm infestation produces anæmia only in the presence of malnutrition.

The anæmia of these recruits improved considerably during the first 3 months of training, but it appears that progress then stopped and much anæmia remained incompletely cured. When a daily dose of ferrous sulphate was given the progress was much more rapid and continued for the whole 5 months of training; even after 3 months most anæmic men were cured.

There are many puzzling features about this universal response to iron therapy. Only 4 per cent of the men who benefited from iron had a typical iron-deficiency anæmia, and only 30 per cent had any of the conventional signs of iron deficiency. Nutrition workers have usually deduced from their analyses that even the poorest Indian diets have an iron content adequate even for the greater needs of women. Three hypotheses come easily to mind to explain these facts.

Firstly, it is possible that a great part of the iron of the poor Indian diet is for some reason not assimilable. Apart altogether from the original chemical form of the iron, workers are only beginning to investigate the influence on iron absorption of mineral balance and such interfering substances as phytic acid.

Secondly, it is possible that the dietary deficiency which is the major cause of this anæmia prevents the utilization of iron. If we replace this factor iron is still necessary to build new hæmoglobin to cure the anæmia.

Thirdly, it is possible that the benefit we observed was not in fact due to iron, but to traces of some other metal present in the ferrous sulphate but deficient in the common Indian diet.

But beyond these speculations one fact remains—most recruits were greatly benefited by a daily dose of ferrous sulphate, and this must be a guide for future policy.

SUMMARY.

1. The hæmoglobin, red cell count, and packed cell volume of some 600 newly joined Indian army recruits were estimated.
2. Most of these men were drawn from the labouring classes, and 25 to 40 per cent of different races had less than 13 g. hæmoglobin.
3. The nursing sepoy were drawn from a higher social class than other recruits, and only 8 per cent had less than 13 g. hæmoglobin.
4. Most of the anæmia was normocytic and normochromic, but every type was found. Macrocytic anæmia was rare.
5. The anæmia of U. P. recruits differed from that of Punjabis and Madrasis.
6. Hookworm infestation was an important contributory cause of the anæmia, but it was often not present in even the severer anæmias, and many infested men were not anæmic.
7. The anæmia of these recruits improved considerably during the first 3 months of training, but progress then stopped and much anæmia remained incompletely cured.
8. When a daily dose of 6 grains ferrous sulphate was given the progress was much more rapid, and even after 3 months most anæmic men were cured.

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CULTIVATION OF A PROTOZOAL PARASITE OF THE CENTRAL NERVOUS SYSTEM *IN VITRO* AND ITS RELATIONSHIP TO RABIES.

BY

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INTRODUCTION.

VEERARAGHAVAN (1944) described a protozoal parasite of the central nervous system of animals suffering from rabies, and discussed the possibility that this parasite might, in fact, be concerned with the aetiology of rabies. The author attempted to propagate this parasite *in vitro* using media, such as NNN medium, ordinarily used for the cultivation of protozoa, and also several synthetic media containing various constituents of the brain, including cholesterol, lecithin, etc., but without success. Later attempts to cultivate this protozoal parasite in a medium similar to that described by Webster and Clow (1936, 1937) for the cultivation of rabies virus, appeared to be successful. In this paper, the results of attempts to cultivate the protozoal parasite are described, and evidence is presented in support of the view that this parasite is the aetiological agent in rabies.

MATERIALS AND METHODS.

A simple liquid medium containing brain extract, serum and fresh normal brain tissue, with or without the addition of some of the constituents of Tyrode solution, was successfully used as a culture medium for the protozoal parasite. A medium prepared from sheep-brain extract and sheep serum gave consistently good results, and this will be described first. The results of experiments using various modifications of this medium will be given later.

Preparation of the medium.—Sheep-brain extract was prepared as follows:—

The brain of a healthy sheep* was dissected out with strict aseptic precautions. A small portion of the brain was weighed and washed repeatedly in sterile distilled water to get rid of all blood. It was then ground up in a mortar with sterile glass, powdered under aseptic conditions, and grinding was continued until a smooth paste was obtained which, when examined under the microscope, was homogeneous and failed to show the presence of intact cells. Ice-cold, sterile distilled water was added gradually in small quantities and grinding continued until a 2 per cent emulsion was obtained. This was allowed to stand for a few minutes to permit the glass-powder to settle, after which the supernatant fluid was centrifuged for half an hour at moderate speed. After centrifuging, the clear supernatant was transferred to a sterile flask, steamed in a steam sterilizer for half an hour on each of three consecutive days, and subsequently tested for aerobic and anaerobic sterility. The 2 per cent extract was diluted to 1 per cent with sterile distilled water at the time of preparation of the medium.

The medium was prepared by mixing one part of sheep serum and two parts of distilled water with six parts of 1 per cent sheep-brain extract prepared as described above. As a rule, 9 ml. of the medium were placed in test tubes, as this facilitated frequent handling of the cultures. When larger quantities were used, flat-bottomed flasks were employed. The medium thus prepared could conveniently be kept for one or two days in cold storage at 4°C.

As mice were not available, the fresh nerve tissue† required for the medium was obtained from a new-born guinea-pig. The animal was killed by drowning and the brain dissected

* It was found to be economical to obtain normal sheep's brain from the heads of freshly slaughtered sheep obtained from the local slaughter-house. This obviated the sacrifice of a whole sheep for each experiment.

† Later work has shown that a ten to twelve-day-old chick embryo brain provides a suitable substitute for young guinea-pig brain.

out under strict aseptic conditions, was transferred to a sterile mortar, washed twice in sterile distilled water, emulsified and diluted in the proportion of two parts of sterile distilled water to one part of brain tissue. Fresh normal brain tissue prepared in this way was added to the sheep-brain extract in the proportion of one drop per ml. just before the medium was inoculated with material containing the parasite. The pH of the medium before inoculation varied from 7.4 to 7.6.

The brains of rabid animals showing the presence of the protozoal body were thoroughly ground with sterile glass-powder and diluted to make a 10 or 20 per cent emulsion, then centrifuged for half an hour at 3,000 r.p.m. The clear supernatant was withdrawn and used as the inoculum. In *in vitro* studies of the protozoal parasite, one part of the clear supernatant from a 20 per cent emulsion was added to nine parts of the medium. For biological titres, the supernatant from a 10 per cent suspension was diluted to 1 per cent with sterile distilled water and one part of the 1 per cent suspension was added to nine parts of the culture medium.

In all experiments to be described, uninoculated medium prepared from sheep-brain extract and fresh guinea-pig nerve tissue was run as a control.

Inoculated media and uninoculated controls were well shaken, nerve tissue allowed to settle and smears made from the supernatants. The cultures were incubated at 37°C. and smears from the supernatants of the cultures were made at intervals of 1, 2, 3, 4, 6, 8, 12 and 24 hours, calculated from the time of inoculation of the test medium. Smears were stained with Giemsa's stain for one hour, dried and examined microscopically for the presence of the protozoal parasite.

Appearances observed in inoculated cultures.—It will be convenient at this stage to describe the appearances seen in inoculated cultures before considering the various modifications of the culture medium which were tried.

Inoculated cultures consistently showed the various forms of the protozoal parasite previously described by the author (Veeraraghavan, 1944). Spores were the commonest forms found in cultures using the medium described above; when they occurred singly it was difficult to distinguish them from particles in the smear, but collections or groups of spores presented a characteristic appearance and were easily recognized. These collections or groups of spores showed considerable variations in shape, dimensions and arrangement; sometimes the spores were closely packed, sometimes loosely arranged; in no case was a capsule observed round the group; individual spores contained in a single group were discrete and comparatively uniform in size, but the size of the spores in different groups varied considerably; some were barely visible under high magnification, while others measured as much as 0.4 microns in diameter; they stained bluish-purple with Giemsa, and could be readily identified in the smears.

Ring forms, growing forms and dividing forms of the parasite were also found, though in small numbers; all stages of development of the parasite might be seen in a single smear.

Spores arranged singly or in groups were visible as early as one hour after inoculation of the culture medium, but were most prevalent after three or four hours. In the six, eight and twelve-hour cultures, spores were present in only small numbers and they were rarely to be found in 24-hour cultures.

Ring forms, growing forms and dividing forms were found in smears of cultures after two to twelve hours' incubation. Frequently the forms seen in the twelve and 24-hour cultures stained black, suggesting that these forms had undergone degenerative changes.

It was not possible to demonstrate the presence of any form of the parasite in smears of the cultures made immediately after inoculation, nor did any of the uninoculated media run as controls show the presence of the parasite.

Modifications of the culture media employed.—The effect of altering the proportions of the various constituents of the medium, as well as the addition of other ingredients to it, were studied. The results were judged by observing the behaviour of the parasite as judged by appearances in stained smears. In certain cases animal experiments were carried

out to confirm the findings observed under the microscope. The results are briefly summarized:—

1. *Brain extract*.—The use of 1 per cent sheep-brain extract with sheep serum gave the best results. When guinea-pig brain extract was used with sheep serum or guinea-pig serum the results were, however, almost equally good. Rabbit-brain extract with sheep serum or with rabbit serum was less satisfactory.

The effect of varying the concentration of brain extract was tried. When trials were made with a medium containing sheep serum and all the constituents of Tyrode solution (except sodium bicarbonate) but in which brain extract was not included a few parasites were found in the cultures after three to eight hours' incubation. In a medium containing six parts of one half per cent brain extract, one part of serum, one part of ten-fold concentration of Tyrode solution (without sodium bicarbonate) and one part of water, the parasites were found in larger numbers. They were seen in greatest numbers when 1 per cent brain extract was used in the above medium instead of half per cent. With 2 per cent brain extract the results were less satisfactory.

Experiments were tried with 1 per cent fresh brain extract with and without steaming, and with brain extract autolysed for 48 hours at 37°C. followed by steaming. As all of them were found to give equally good results, steaming of the fresh brain extract was regularly adopted to ensure sterility. It was found that satisfactory results could be obtained with brain extract kept for two or three weeks in cold storage.

2. *Serum*.—The effect of adding various sera to the medium was tried. Sheep serum and guinea-pig serum gave equally good results. Horse serum was tried in only one experiment and gave good results. Human serum and rabbit serum were less satisfactory. When serum capable of neutralizing *fixed virus in vitro* (collected from patients who had undergone antirabic treatment) was added to the medium, the parasites were not to be found.

The effect of altering the concentration of the serum in the medium was tried. When serum was not added to the medium, the parasites were not encountered. When the medium contained 5 per cent concentration of sheep serum, the parasites were found in large numbers. A 10 per cent concentration of serum in the medium was considered the optimum and appeared to give better results than serum concentrations of 15 or 20 per cent.

3. *Tyrode*.—Tyrode solution was prepared in two parts separately. The first part (Tyrode I) contained NaCl 8 g., KCl 2 g., CaCl₂ 0.2 g., MgCl₂ 0.1 g. and NaH₂PO₄ 0.05 g. in 100 ml. of distilled water.

The second part (Tyrode II) contained glucose 1 g. and NaHCO₃ 1 g. in 100 ml. of distilled water. Thus, the solutions were ten times concentrated. These solutions were filtered through Seitz pads, tested for sterility and stored separately.

When one part of Tyrode I and one part of Tyrode II were added to a medium containing six parts of 1 per cent sheep-brain extract and one part of sheep serum, the pH of the medium varied from 8.0 to 8.2. The smears of the cultures stained dark-blue with Giemsa's stain, rendering observation of the parasite difficult. When sodium bicarbonate was omitted and only one part of 1 per cent glucose was added in place of Tyrode II, the ring forms, growing forms and dividing forms of the parasite were encountered in large numbers. Collections of spores were found only occasionally. When glucose alone was added to the medium without the Tyrode I, the spores were found in large numbers, but the other stages were encountered only in small numbers. The addition of various concentrations of glucose to the cultures seemed to make little difference, although varying amounts (0.1 to 2.0 per cent) were used:

4. *Fresh nerve tissue*.—Attempts to cultivate the parasite in the above media without the addition of fresh nerve tissue proved to be unsuccessful. Evidence of multiplication of the parasite was obtained only when fresh nerve tissue from a guinea-pig, one or two days old, was added to the medium immediately before inoculation. The results were not satisfactory when nerve tissue from adult guinea-pigs was used.

Experiments with the brains of healthy animals.—The clear supernatants from the suspensions of the brains of healthy animals were inoculated into culture media and smears examined for the presence of the parasite. Culture media inoculated with the brain suspensions of fifteen dogs, twelve guinea-pigs, six rabbits and four sheep, which showed no

evidence of rabies infection, did not show the presence of the parasite. Sections of the hippocampus major, cerebellum and mid-brain of all these animals showed neither the parasite nor Negri bodies.

Experiments with strains of rabies virus isolated from naturally infected dogs and jackals showing the presence of the parasite.—The jackal (J) strain and dog (D) strain described in the previous paper (Veeraraghavan, 1944) and six other strains of street virus, four of which were isolated from dogs and two from jackals, were studied. Parasites and Negri bodies were observed in the brains of guinea-pigs inoculated with these strains. The various stages of the parasite were regularly found in the cultures of the brains of these guinea-pigs. These cultures were inoculated subdurally into guinea-pigs; all of them contracted rabies and died; the parasite was found in their brains and in cultures prepared from these.

Experiments with other strains of street virus.—The parasite, in all its stages, was observed regularly in cultures inoculated with the supernatants from the suspensions of the brains of a number of rabid animals (twenty-five dogs, two cats, one goat and one bull-calf) sent to this Institute for examination. Sections of the brains of all these animals showed Negri bodies. The utilization of the technique of cultivation as a rapid method for the diagnosis of rabies in animals is described in another paper (Veeraraghavan, 1945).

Experiments with Paris strain of rabies fixed virus.—All stages of the parasite were demonstrable in cultures inoculated with the Paris strain of rabies *fixed* virus maintained at the Pasteur Institute, Coonoor, as well as the strain of virus maintained at the Central Research Institute, Kasauli, and the Haffkine Institute, Bombay. The schizont stages of the parasite and a few Negri bodies were found in the brains of guinea-pigs inoculated with the culture virus. The parasite was found in cultures inoculated with the brains of these animals.

Filtration experiments.—

1. *Jackal (J) strain virus.*—The virus in this experiment was obtained from the brain of a guinea-pig which was completely paralysed after subdural inoculation with a 6-hour culture of the jackal (J) strain virus. The parasite was found in the brain of the guinea-pig. The clear supernatant from a 10 per cent suspension of the brain after centrifugalization was filtered through Berkefeld V and N candles. Smears of the filtrates did not show any forms which could be definitely identified. The filtrates were inoculated into culture media and into guinea-pigs. The cultures, after two hours' incubation at 37°C., showed the presence of the parasite. Guinea-pigs inoculated with the V and N filtrates immediately after filtration and after six hours' cultivation in the culture media died of paralytic rabies. The parasite and Negri bodies were found in the brains of all these animals. Filtration through a Berkefeld W candle could not be tried as a good candle was not available.

2. *Paris strain of rabies fixed virus.*—(a) The above experiment was repeated with the Paris strain of rabies *fixed* virus. Guinea-pigs inoculated with the Berkefeld V filtrate immediately after filtration and with cultures of the V filtrate showed signs of paralytic rabies and died. Two guinea-pigs inoculated with the Berkefeld N filtrate immediately after filtration escaped, while the animals inoculated with the cultures of the Berkefeld N filtrate died of rabies. The schizont stages of the parasite and a few Negri bodies were encountered in the mid-brains of the guinea-pigs inoculated with the culture virus. Cultures inoculated with the brains of these animals showed the presence of the parasite.

(b) Filtration experiments were tried with the Paris strain of rabies *fixed* virus after cultivation in artificial culture. The virus was obtained from a guinea-pig which had died as a result of infection with 1 in 80,000 dilution of a 12-hour culture of the *fixed* virus used in another experiment. Two ml. of the clear supernatant from a 20 per cent suspension of the brain of the above guinea-pig were inoculated into eighteen ml. of the culture medium containing brain extract and sheep serum. Smears of the cultures showed the presence of the parasite. The 11-hour culture was lightly centrifuged and the clear supernatant withdrawn. This was divided into two equal parts. One part was filtered through Berkefeld V candle and the other through Berkefeld N candle. Smears of the filtrates showed minute dots which were difficult to identify. The filtrates were inoculated into fresh culture media and into guinea-pigs. Smears of the cultures of the filtrates showed the presence of the parasite. Guinea-pigs inoculated with the Berkefeld V and N filtrates of the culture virus developed

paralytic rabies and died. A few schizont stages of the parasite were encountered in the mid-brains of the animals.

Titration experiments.—

1. *Jackal (J) strain.*—The evidence of multiplication in cultures was obtained by comparing the highest dilutions in which the inoculum and the cultures proved to be infective. when inoculated subdurally into guinea-pigs. Nine ml. of the culture media were inoculated with one ml. of the clear supernatant from a 1 per cent suspension of the brain of a guinea-pig showing the presence of the parasite. Various dilutions of the inoculum were given to guinea-pigs. About half ml. of the supernatant was withdrawn from the 4-, 8-, 12-, 24- and 48-hour cultures. diluted with sterile distilled water and 0.2 ml. of various dilutions inoculated subdurally into guinea-pigs. A typical titration with the jackal strain virus is given in Table I:—

TABLE I.

Titration experiments with cultures of jackal (J) strain virus.

Number of hours at 37°C.	CULTURES INOCULATED SUBDURALLY IN 0.2 ML. AMOUNTS INTO 3 GUINEA-PIGS IN DILUTIONS:—														
	1/1,000			1/5,000			1/10,000			1/20,000			1/40,000		
0	*6/8	7/9	7/9	7/10	S	S	S	S	S	S	S	S	S	S	—
4	7/9	7/9	8/10	9/10	9/11	S	S	S	S	S	S	S	S	S	—
8	—	—	—	9/11	9/12	9/12	S	S	S	S	S	S	S	S	—
12	—	—	—	8/10	9/11	9/12	8/11	9/11	10/12	11/13	S	S	S	S	S
24	8/11	9/11	9/12	9/11	S	S	S	S	S	S	S	S	S	S	—
48	10/12	S	S	S	S	S	S	S	S	S	S	S	S	S	—

Note.—All dilutions are calculated on the basis that the undiluted culture represents a 1 in 1,000 dilution of the virus.

*6/8 = Guinea-pig paralysed on the sixth day and died on the eighth day.

S = Guinea-pig remained well.

— = Dilution not tested.

The results indicate that the highest concentration of the parasite was reached in the 12-hour culture.

2. *Paris strain of rabies fixed virus.*—A series of titration experiments were carried out with cultures of the Paris strain of rabies *fixed* virus. When fresh brain tissue was omitted from inoculated cultures containing brain extract and serum with or without glucose and Tyrode I. there was no evidence of multiplication of the virus. On the contrary, the virus content of the cultures appeared to diminish steadily and after 24 to 48 hours undiluted cultures were not infective.

Titration experiments were carried out with media containing brain extract and fresh nerve tissue, but without the addition of serum. The virus was not demonstrable in the cultures after 24 hours indicating that serum is necessary for its survival and multiplication.

Cultures of *fixed* virus in media containing 1 per cent sheep-brain extract and 10 per cent sheep serum have given consistently good results. The results of a typical experiment are given in Table II.

TABLE II.

Titration experiments with cultures of Paris strain of rabies fixed virus.

Number of hours at 37°C.	CULTURES INOCULATED SUBDURALLY IN 0.2 ML. AMOUNTS INTO 3 GUINEA-PIGS IN DILUTIONS:—									
	1/1,000	1/10,000	1/20,000	1/40,000	1/80,000	1/100,000				
0	—	*6/8 6/8 6/9	S S S	S S S	—	—				
4	—	6/8 6/9 7/9	7/9 7/10 8/10	8/10 S S	S S S	—				
8	—	—	7/9 8/9 8/9	10/12 10/13 11/13	S S S	S S S				
12	—	—	8/9 8/9 9/10	8/10 9/10 9/10	7,8 7/9 8/10	8/11 S S				
24	8/10 9/11 9/11	9/10 9/12 9/12	10/12 S S S	S S S	—	—				
48	9/11 9/12 S	10/13 S S	S S S	S S S	—	—				

Note.—All dilutions are calculated on the basis that the undiluted culture represents a 1 in 1,000 dilution of the virus.

* 6/8 = Guinea-pig paralysed on the sixth day and died on the eighth day.
S = Guinea-pig remained well.

— = Dilution not tested.

It will be seen from Table II that the concentration of the virus increased steadily up to 12 hours, after which it steadily decreased. The highest concentration of the virus obtained in cultures was eight times that present in the original inoculum.

Experiments with rabies-neutralizing serum.—When serum capable of neutralizing *fixed* virus *in vitro*, collected from patients who had undergone antirabic treatment, was added to the cultures of *fixed* virus and the jackal (J) strain virus, the parasites were not found in smears of the cultures.

One ml. of a 4-hour culture of rabies *fixed* virus showing the presence of the parasite was mixed with one ml. of rabies-neutralizing serum and incubated at 37°C. for two hours. The mixture was inoculated into guinea-pigs, all of which remained alive and well. On the other hand, the culture virus diluted with normal human serum was found to be infective.

DISCUSSION.

It will be clear from the observations recorded that it is possible to cultivate the protozoal parasite of the central nervous system described by the author (Veeraraghavan, 1944) *in vitro* in a simple liquid medium containing sheep-brain extract, sheep serum and fresh young guinea-pig brain tissue. When fresh nerve tissue was omitted from the medium, cultures inoculated with the parasitic material did not show the presence of the parasite and proved to be avirulent within twenty-four to forty-eight hours indicating that fresh nerve tissue is essential for the survival of the parasite. In the medium containing sheep-brain extract and sheep serum, the spore forms of the parasite were most commonly seen, while other stages were found only in small numbers. The addition of glucose to the medium did not appear to influence the prevalence of the various forms observed, although various concentrations were tried. But, when Tyrode solution without sodium bicarbonate was added to the medium, spore forms were found only in small numbers, while other forms predominated. The morphological appearances of the parasite and the method of multiplication were those of a protozoan.

The evidence of multiplication of the parasite in the medium is based on the following observations: (1) Smears of uninoculated culture media incubated at 37°C. did not show the presence of the parasite. The parasite was not found in smears of the media immediately after inoculation with the clear supernatants from centrifuged suspensions of the parasitic material, but all the stages of the parasite were demonstrable in incubated cultures. (2) The cultures showing the presence of the parasite were infective in much greater dilution than the original inoculum when titrated in animals. The only other explanation of these findings which might be considered is that, following manipulation in cultures, the virus may, as the result of lysis of the brain cells, become more readily 'available' or more invasive in character.

The exact mode of multiplication of the parasite in the medium is not clear. The spore forms of the parasite were occasionally found in the cytoplasm of nerve cells of the guinea-pig inoculated into the medium. But the other forms of the parasite were seldom found within the cytoplasm of the nerve cells. These observations suggest that the spores might undergo intra-cellular proliferation and be set free in the medium as a result of the disintegration of the cell. The further development of the parasite might take place in the medium. On the other hand the appearance of the groups of spores in the supernatants of the cultures as early as an hour after inoculation suggests the possibility that the development of the parasite might take place in the medium itself outside the nerve cells. The presence of living cells in the medium, however, appears to be essential.

The following evidence indicates that the protozoal parasite is the aetiological agent in rabies: (1) The parasite was not demonstrable in cultures inoculated with suspensions of the brains of guinea-pigs, rabbits, sheep and dogs which showed no evidence of rabies infection. (2) The parasite was regularly found in cultures inoculated with the strains of rabies virus isolated from dogs and jackals showing the presence of the parasite. Guinea-pigs inoculated with the cultures died of rabies and the parasite was demonstrable in their brains. Cultures inoculated with suspensions of the brain of these animals showed the presence of the

parasite. (3) The cultures of Berkefeld V and N filtrates of some strains of virus isolated from dogs and jackals showed the presence of the parasite. The parasite was also demonstrable in the brains of animals which died as a result of inoculation with the cultures. (4) The parasite was regularly encountered in cultures inoculated with any strain of *street* virus. (5) The parasite was demonstrable in cultures inoculated with the Paris strain of rabies *fixed* virus maintained at this Institute as well as the same strain of virus maintained at other institutes in India. The virus after cultivation in the medium was infective in much higher dilution than the original inoculum when titrated in animals. The schizont stages of the parasite and Negri bodies were encountered in the mid-brains of guinea-pigs inoculated with the culture virus. (6) Cultures of the Berkefeld V and N filtrates of the Paris strain of rabies *fixed* virus showed the presence of the parasite. The inoculation of the cultures of the filtrates into animals reproduced the infection. In some instances the filtrates immediately after filtration did not prove to be infective but after cultivation were found to be infective. (7) The cultures of the Paris strain of rabies *fixed* virus showing the presence of the parasite, after filtration through Berkefeld V and N candles, proved to be infective. The parasite was demonstrable in cultures inoculated with the filtrates. The schizont stages of the parasite and Negri bodies were found in the mid-brains of animals inoculated with the filtrates. (8) The parasite was not found when known rabies-neutralizing serum was added to the culture medium. Cultures of the Paris strain of rabies *fixed* virus showing the presence of the parasite were not infective to guinea-pigs after being mixed with serum known to neutralize rabies virus and incubated for two hours at 37°C.

SUMMARY.

1. A simple medium containing sheep-brain extract, sheep serum and fresh young guinea-pig brain has been described for the *in vitro* cultivation of the protozoal parasite of the central nervous system previously described by the author.

2. The morphological appearances of the parasite and the method of multiplication *in vivo* and *in vitro* are those of a protozoan.

3. Cultures showing the presence of the parasite were found to be infective in much higher dilution than the original inoculum.

4. The parasite has never been encountered in the brains of normal guinea-pigs, rabbits, sheep and dogs, or in cultures of them.

5. The parasite has been observed regularly in cultures inoculated with the Paris strain of rabies *fixed* virus and with all of the strains of rabies *street* virus so far investigated.

6. Filtration experiments with Berkefeld V and N candles have been described; these experiments indicate that certain stages in the development of the parasite in cultures are 'filtrable'.

7. These findings provide further evidence in support of the view that the protozoal parasite described is connected with the aetiology of rabies.

ACKNOWLEDGMENTS.

The author desires to express his thanks to Lieut.-Colonel K. R. K. Iyengar, C.I.E., I.M.S. (*retd.*), Director, Pasteur Institute, Coonoor, for his valuable guidance and kind encouragement. He is deeply indebted to Lieut.-Colonel H. W. Mulligan, I.M.S., Director, Central Research Institute, Kasauli, for his kind help and advice. His thanks are due to his Laboratory Assistants, Mr. M. B. Ajjah and Mr. A. Kulla, for the technical help received.

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Addendum.—Later work has shown that the following medium can be successfully used for the cultivation of the protozoal parasite *in vitro* with better results. Sheep-brain extract (1.0 per cent), 60 ml.; glycine (1.5)

per cent), 10 ml.; peptone (1.5 per cent), 10 ml.; sheep serum, 1 ml.; and distilled water, 9 ml. Just before inoculation of the medium with the virus an emulsion of the brain of one guinea-pig in 5 ml. of distilled water is added to every 45 ml. of the medium. With the above medium all the stages of the parasite can be demonstrated in cultures of the Paris strain of rabies *fixed* virus and other strains of rabies *street* virus so far investigated. The concentration of the rabies *fixed* virus obtained in cultures, using the above medium, is much higher than that reported in the paper. A dilution of 1 in 450,000 of the culture virus proved to be readily infective in guinea-pigs compared with the dilution of 1 in 80,000 previously recorded. The utilization of this method of cultivation for the preparation of a culture vaccine is in progress.

N. VEERARAGHAVAN.

22nd September, 1945.

A RAPID METHOD FOR THE DIAGNOSIS OF RABIES IN ANIMALS.

BY

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INTRODUCTION.

ALTHOUGH the occurrence of Negri bodies in the brain is regarded as definite evidence of rabies infection, failure to demonstrate these bodies, while providing strong presumptive evidence against rabies, leaves the diagnosis in doubt and necessitates the issue of an inconclusive report. The result of the biological test with the suspected rabid material may not be known before three months and, therefore, proves to be of little or no value in assessing the need for the treatment of bitten persons. It is evident that a rapid and reliable method for the diagnosis of rabies in animals would satisfy a need of primary importance.

A protozoal parasite of the central nervous system was described by Veeraraghavan (1944) and the possibility of the parasite being connected with the aetiology of rabies was discussed. Veeraraghavan (1945) succeeded in cultivating the parasite *in vitro* in a simple liquid medium containing brain extract, serum, and fresh nerve tissue. The various stages of the parasite were never observed in cultures of the brains of healthy animals, but were regularly demonstrable in cultures of the brains of animals which died of natural or experimental rabies infection. The possibility of utilizing the technique of cultivation in the rapid diagnosis of rabies was investigated. The results, which have been found to be satisfactory, are given in this paper.

MATERIALS AND METHODS.

The routine procedure adopted at the Pasteur Institute, Coonoor, for the diagnosis of rabies is as follows: A portion of the brain of the animal suspected to be rabid is sent to the Institute in a solution containing three per cent potassium bichromate and half per cent glacial acetic acid, and another portion is sent in sterile fifty per cent glycerine. Owing to the present difficulty of obtaining supplies of glycerine, veterinary surgeons have been requested to send specimens for biological test in half per cent carbol-saline instead of fifty per cent glycerine. The preservation of rabid brain tissues in half per cent carbol-saline was suggested by Olah (1938), and has been found to be quite satisfactory. Specimens sent in the acetic-bichromate solution are almost fixed by the time they reach the Institute from out-stations. A thin slice of the hippocampus major is crushed under a coverslip and examined. Negri bodies, if present, can be readily identified within the cytoplasm of the nerve cells. If the brain is positive, further examination is not carried out. If Negri bodies are not found, a portion of the hippocampus major is dehydrated, cleared, embedded in paraffin, sectioned, stained with Mann's stain overnight and examined for the presence of Negri bodies. For the biological test, which is carried out only when Negri bodies are not found, a suspension of the brain of the animal in glycerine or carbol-saline, if not highly decomposed, is inoculated into the neck muscles of a guinea-pig and the animal kept under observation for a period of three months. If the animal dies during the observation period, its brain is examined for the presence of Negri bodies. But, if the animal remains well at the end of three months the result of the biological test is reported as negative.

The above routine procedures were carried out in parallel with the cultural method to be described. The medium and the method of cultivation were as described by the author (1945). The medium contained six parts of one per cent sheep-brain extract, one part of sheep serum and two parts of distilled water. Guinea-pig-brain extract with guinea-pig or sheep serum

may also be used. Eight ml. of the medium were placed in an ordinary medium-sized test-tube. Just before inoculation, ten drops of an emulsion of fresh nerve tissue obtained from a young guinea-pig, one or two days old, were added to the medium. A twenty per cent emulsion of the brain of the animal suspected to be rabid was centrifugalized for half an hour at 3,000 r.p.m. and two ml. of the clear supernatant inoculated into the medium. It was found that 0.4 ml. of the clear supernatant inoculated into 1.6 ml. of the culture medium gave equally good results. An uninoculated medium, a medium inoculated with the brain of an animal known to be rabid and another inoculated with the brain of a healthy animal were always run as controls. The inoculated media and the uninoculated control were shaken well, the nerve tissue allowed to settle down, and smears made from the supernatants. The cultures were then incubated at 37°C. Smears from the supernatants of the cultures were made after one, two, three, four, six and eight hours. After making the smears the cultures were shaken well and replaced in the incubator. In practice it was found that the examination of the smears of the 6- and 8-hour cultures were seldom necessary. The smears were stained with Giemsa's stain for one hour, dried and examined under the microscope.

RESULTS.

The various forms of the parasite encountered in cultures of rabid brains has already been described by the author (1945). Collections of spores were found as early as one hour after incubation. They were found in largest numbers in smears of the 3- or 4-hour cultures. The other stages of the parasite were seen in comparatively smaller numbers in cultures using the above medium.

Smears of the uninoculated media run as controls did not show the presence of the parasite.

Culture media inoculated with the brain suspensions of fifteen dogs, twelve guinea-pigs, six rabbits and four sheep, which showed no evidence of rabies infection, did not show the presence of the parasite. Negri bodies were not found in the hippocampus major, cerebellum and mid-brain of these animals.

The brains of twenty-five dogs, two cats, one goat and one bull-calf, suspected to have died of natural rabies infection, were studied. Among these Negri bodies were found in crushed specimens of the hippocampus major in twelve dogs, two cats, one goat and one calf. Negri bodies were seen only after sectioning the hippocampus major in six dogs. The brains of five dogs did not show the presence of Negri bodies and were reported as inconclusive: out of these, the biological test showed the brains of four dogs to be positive for rabies. The brains of two dogs were highly decomposed and unsuitable for reliable microscopical examination and biological test.

Using the technique of cultivation described, the following results were obtained with the above brains:—

1. The brains of all the animals, which were declared to be positive for rabies infection after examination of either crushed specimens or sections, showed the presence of the parasite in cultures.

2. Out of the five dog brains, which were declared to be inconclusive after microscopical examination of sections, four proved to be positive by culture: the presence of rabies infection in those 4 dogs was later confirmed by the biological test. -

3. The brains of two dogs, which were highly decomposed and reported upon as unsuited for examination, proved to be positive by culture. The centrifugalization of the decomposed brain for half an hour seemed to deposit most of the bacteria, so that they were rarely encountered in smears of the cultures up to the fourth hour. However, the 6- and 8-hour cultures generally showed the presence of bacteria, their number having increased after this longer period of incubation. The culture method renders the examination of even grossly decomposed brains possible. A summary of the results is shown in the Table.

TABLE.

Diagnosis of rabies in animals compared by methods commonly used and by culture.

Serial No.	Name of animal.	Number of animals whose brains were studied.	Nature of rabies infection.	Nature of virus.	Number in which direct examination of brain was positive for rabies.	Number in which direct examination was negative but section positive.	Number in which section was inconclusive but biological test positive.	Number in which section was inconclusive but biological test negative.	Number in which brain was unfit for examination.	Number in which brain was positive by culture.	Number in which brain was negative by culture.
1.	Dogs	... 25	Natural	<i>Street virus</i>	12	6	4	1	2	24	1
2.	Cats	... 2	"	"	...	2	2	...
3.	Goat	... 1	"	"	1	1	...
4.	Bull-calf	... 1	"	"	1	1	...
5.	Guinea-pigs	... 6	Experimental	"	6	6	...
6.	Rabbits	... 6	"	<i>Fixed virus</i>	6	...
7.	Sheep	... 6	"	"	6	...

The brains of animals which died of experimental rabies infection were studied. The cultures of the brains of six guinea-pigs, which died as a result of infection with various strains of rabies *street virus*, showed the presence of the parasite. The parasite was encountered in cultures of the brains of six rabbits and six sheep inoculated with the Paris strain of rabies *fixed virus*.

DISCUSSION.

The technique of cultivation described affords a simple and rapid method for the diagnosis of rabies in animals, the result being obtained within a few hours. The method appears to be as delicate as the biological test, the results of which may not be known for as long as three months. With the technique described, it is possible to examine brains which are grossly decomposed and unsuitable for microscopical examination or biological test.

SUMMARY.

A rapid and delicate method for the diagnosis of rabies in animals is described.

ACKNOWLEDGMENTS.

The author desires to express his thanks to Lieut.-Colonel K. R. K. Iyengar, C.I.E., I.M.S. (*retd.*), Director, Pasteur Institute, Coonoor, for his valuable guidance and kind encouragement. He is deeply indebted to Lieut.-Colonel H. W. Mulligan, I.M.S., Director, Central Research Institute, Kasauli, for his kind help and advice. His thanks are also due to his Laboratory Assistants, Mr. M. B. Ajjah and Mr. A. Kulla, for the technical help received.

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THE SUSCEPTIBILITY OF INDIANS TO CANCER.

BY

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SEVERAL observers have remarked that there is considerable variation both in the total incidence and in the types of cancer in different parts of the world. It may, therefore, be expected that geographical differences may reveal a disproportionately high incidence of cancer in some races and relative immunity in others. If such differences could be shown to be real and not apparent, they may suggest lines for a further study of the habits of the people and their environment. Unfortunately, much of the laboriously gathered material on this point suffers from shortcomings which render it unsuitable for detailed analysis. In many cases the investigators have attempted to fit their facts into preconceived opinions. For instance, an idea had gained wide currency that cancer is a disease of the civilized races or the inhabitants of Western and Middle Europe. The European observers, therefore, could see very little cancer in the rest of the world, as compared to Europe; and even among themselves they discovered more cancer among their own countrymen. Many theories on the nature and causation of cancer have thus emanated from ideas based on prejudices.

There are two principal sources of information on the prevalence of cancer in India. The first is derived from the experience and impressions of medical men attached to hospitals in different parts of the country. This information would be valuable if the experience of these men had extended over a period of years and particularly if they have had previous experience in institutions abroad, where cancer is studied and treated more carefully. In this country the data obtained from different hospitals are often contradictory and sometimes conflicting. Megaw and Gupta (1927), in an attempt to discover the geographical distribution of some of the diseases of India, collected some quaint information regarding cancer. For instance, 51 out of 202 civil surgeons reported that cancer of the skin was unknown in their locality. Seventeen made a similar statement regarding breast, 30 for uterus, 120 for stomach and 43 for cancer of the mouth (one of the commonest types of cancer here). These are very surprising statements, but a visit to the clinics in mofussil hospitals would convince any impartial observer that most of the out-patient work is very perfunctory and that medical officers have hardly any opportunity of seeing, much less examining carefully, most of their hospital patients. During a recent tour we had the opportunity of seeing medical men in district hospitals examining, recording and prescribing single-handed for nearly 300 patients in the course of a morning.

The missionary doctors spend more time over their patients, but unhappily the meagre diagnostic facilities imposed upon them by 'the dictates of the budget' do not permit a pathological examination of the suspected materials. It is more than likely that several cases of malignant disease of the inaccessible and intermediate regions of the body are missed by them. However, the experience of mission doctors at Kashmir, Miraj, Neyoor, Wai and Vengurla suggests that they see 'much cancer in their patients' (Hoffman, 1936).

More reliable information is obtainable from the big institutions in the country which undertake the teaching of undergraduate and post-graduate students in medicine. The opinions of three well-known teachers from widely separated regions may be cited in this connection: Bradfield (1930) from the Madras Medical College states 'formerly we have commented on the comparative rarity of cancer of the breast, but during the past year this belief has been upset and suggests that there is a tendency for women to conceal the disease. . . . The statistics bear out, what has been reported before, that the incidence of malignant disease is very much the same in South India, as it is in other parts of the world'. Vishwa Nath and Grewal (1933) from King Edward Medical College, Lahore, state that 'The malignant disease is not uncommon in India . . . considering that hospitals with in-patient accommodation in India are few and far between, that morbidity in the country as a whole is high, and that only

a small proportion of the sick attend the hospitals, it will not be an unwarranted speculation to state that the incidence of cancer in India stands at a figure not far removed from its incidence in the West'. The impressions of Leonard Rogers (1925) regarding the incidence of cancer in Calcutta are summarized in his Finlayson lectures delivered at Glasgow in 1925. He was of opinion that 'malignant tumours, including both connective tissues and epithelial types, are about equally common in Bengal and England, with a slight excess in the tropical country'. This opinion of his was based on a long and brilliant career as a physician and pathologist at the commencement of this century in Bengal; and on a comparison of 1,067 post mortems at the Calcutta Medical College Hospital with 1,000 post mortems at St. Mary's Hospital in London. His analysis showed an autopsy rate of 4.59 per cent in Calcutta as compared with 13.8 per cent in the London records. The difference in the proportion he thought could be partly accounted for by the comparatively lower age of his cases. Our own impressions are based on an experience of nearly twenty years' work in Bombay and of five years in the Department of Pathology in the University College Hospital, London. We are in essential agreement with the opinions of the observers in Madras, Lahore and Calcutta. However, as human impressions are apt to be misleading it is better to have them tested by the available statistical information. Accordingly, we have analysed the autopsy records of the King Edward Memorial Hospital, Bombay, during the last ten years.

The hospital which was opened in 1926 has an average annual in-patient attendance of 13,045 and an out-patient attendance of 618,266. This hospital along with the Nowrosjee Wadia Maternity Hospital and the Bai Jerbai Wadia Children's Hospital, each with a complement of nearly 150 beds, is situated in the heart of the industrial portion of the city and constitutes the largest medical centre in the north of the island. The Tata Memorial Hospital for the treatment of malignant diseases was opened in 1941 in the immediate vicinity of this group of hospitals. Its presence may have resulted in a slight diminution of the cancer cases of accessible regions at the K. E. M. Hospital. This short description of the K. E. M. Hospital is necessary to furnish a background against which the post-mortem data can be reviewed.

TABLE I.

King Edward VII Memorial Hospital, Bombay (1934-43).

Total deaths	12,763
Males	9,356 (73.30 per cent)	
Females	3,407 (26.70 " ")	
Total autopsies	3,919
Males	3,196 (81.55 " ")	
Females	723 (18.49 " ")	
Tropical disease	341 (8.69 per cent).
Males	296 (86.79 " ")	
Females	45 (13.19 " ")	
Malignant disease	158 (4.41 " ")*
Males	110 (69.60 " ")	
Females	48 (29.80 " ")	
Clinical diagnosis of cancer	105
No cancer at autopsy (C)	16 (15.24 " ").
Autopsy diagnosis of cancer	158
Cancer not diagnosed (O)	69 (43.04 " ").

* After omitting tropical diseases from total autopsies.

(C) Errors of commission.

(O) Errors of omission.

During the ten-year period 1934-43 there were 12,763 deaths at the hospital and an autopsy examination was carried out on 3,919 (30.74 per cent). The proportion among the total dead was 2.8 males to 1 female, and among the autopsied 2.3 to 1. There was thus no disproportionate selection of any particular sex. Out of the 3,919 autopsies which were performed either under my personal direction or that of my successor, there were 341 cases which could be classified as due to tropical diseases and 158 as due to malignant disease or cancer. It is of interest that the proportion of cancer deaths to total deaths, after omitting those due to tropical disease, is 4.41 per cent as against 4.59 per cent mentioned by Leonard Rogers for the Calcutta Medical College Hospital on the basis of 1,600 post-mortem examinations. This proportion closely approximates the number of cancer autopsies (271 out of a total of 5,963 during the years 1926-36) reported from the Pathological Institute of Belgrade University by Miletitch (1938), giving a percentage of 4.56. In presenting these data we are aware that the incidence of any particular disease in a locality cannot be determined on the basis of the experience of an individual or a group of medical institutions. 'Every hospital, to a certain extent, is a specialized institution, and with regard to cancer, attracts certain types of malignant disease more than others on account of special facilities for treatment' (Hoffman, *loc. cit.*). This is particularly noticeable in the autopsy material, as patients grievously ill with cancer of accessible regions of the body prefer to go home and die when they or their relatives realize that a cure would not be possible. There is also a further selection by the medical men who avoid admitting, as in-patients, persons who are too far advanced for any effective treatment, and who, if admitted, would occupy much needed beds for a long period of time. It is our unfortunate experience that many cases are seen in a very late stage of disease and do not find a place in hospital records. The hospitals attached to medical colleges, however, possess certain advantages for a study of this type: (1) They have at their disposal most of the modern means for accurate diagnosis such as biochemical tests, tissue investigation, endoscopic exploration and x-ray examination. The accuracy of clinical and autopsy diagnosis of the common types of cancer would, therefore, be approximately the same at all these places. (2) As these institutions impart basic instruction to medical students they strive to admit as large a variety of cases as possible. They, therefore, tend to represent a fairly accurate cross-section of the disease in that locality. The data collected from an isolated institution thus become much more valuable when they are studied along with information from similar institutions in the same or other countries.

The second source of information is the vital statistics of the country. When one attempts to investigate the frequency of cancer in India on the basis of the vital statistics published by official agencies one immediately encounters serious difficulties. There is plenty of information on matters such as individual institutions, medical services, disease and pestilence, although very little comparable and well co-ordinated data are available about the general health of the people. It is evident that, in order to obtain a reliable estimate of the health conditions of a people, it is necessary to obtain sufficiently accurate facts on the following points: (1) The population of a given locality at any particular time, (2) the number of births during each succeeding period, (3) the number of deaths, and the sex and age composition, and finally (4) a reasonably accurate statement about the cause of death.

Information regarding population is available from the decennial Census Reports of India. This information may be regarded as being fairly reliable for the principal cities during the Census of 1921 and not so reliable for 1931. The recorded number of births and deaths is also probably accurate for the big cities but inaccurate in outlying districts of Sind, N.-W. F. Provinces and Assam. The main difficulty, however, lies in discovering the real cause of death. Both in the urban and in the rural areas the nature of the official organization is such that it is difficult to get accurate information about the cause of death in most cases. The conditions, except in a few big centres in India, are reminiscent of those obtaining in the 17th century in Europe. At that time the London Bills of Mortality were compiled by parish clerks who also entered the cause of death which was discovered by official searchers. They were generally ignorant of even the elementary notions of medicine. In most parts of India even to-day the village watchman (chowkidar or kotwal) 'is the diagnostician who reports to the nearest police station once or twice a month the deaths occurring in his area and the causes as he knows them. It is this information which is the foundation of Indian Vital Statistics' (Vishwa Nath, Lall and Singh, 1933). Two factors noticeably diminish the value of the records of death

from cancer in most countries but particularly so in India. The first is the inherent difficulty of diagnosing cancer correctly in many cases even by trained medical men. Most types of cancer are diseases of slow and insidious evolution and do not obtrude upon a superficial observer by characteristic symptomatology. This is very well shown by the records of some institutions where the cause of death was later verified at autopsy. In the 1,249 cancer autopsies out of a total of 8,500 studied by de Vries (1927) in Amsterdam, malignant disease was not diagnosed in 249 (20 per cent) and was wrongly diagnosed in 102 cases, in which no cancer was found at autopsy. Similarly, in the 3,712 autopsies performed by Wells (1923) in Chicago, there were 545 cases of malignant disease of which 178 had not been diagnosed clinically. The errors of omission and commission in the 3,919 autopsies studied by us is shown in Table I. The opinion of Wells in this connection is worth reflecting upon. 'When we find diagnostic errors ranging from 25 to 40 per cent in patients who have been examined in modern hospitals in Germany, England and America, with the advantage of exploratory operations, roentgen rays and laboratory studies under the most competent medical men in the community, it is certain that the diagnostic errors made throughout the country at large must be even greater'. This clearly indicates the degree of importance to be attached to the cancer mortality rates of 20 to 25 per 100,000 living population (Hoffman, *loc. cit.*) returned by different medical officers of health in some of the principal cities in this country, in most cases without an autopsy or a detailed clinical examination.

The second factor for consideration which reduces the value of official data is that Indian Vital Statistics ignore the existence of cancer, 'because the disease has not yet come within the cognizance of village chowkidar who reports on causes of death in rural areas, and in urban areas the certification of death by registered medical practitioners has not yet become obligatory' (Vishwa Nath and Grewal, *loc. cit.*). Fortunately for this inquiry the Administrative Reports of the Municipal Commissioner of the City of Bombay (1931-40) give a tabulated statement of the number of certificates of the cause of death received from different persons; and the percentage of total certificates of death received, to the total reported mortality. It appears from Table II that only 37.56 per cent of all deaths in Bombay are certified by persons who have any acquaintance with medicine. It will also be evident that all deaths reported as being due to cancer in the city of Bombay must be in the group of certified deaths.

TABLE II.
Certification of causes of death.

Year.	Total deaths.	NUMBER OF CERTIFICATES OF THE CAUSE OF DEATH RECEIVED FROM						Percentage of total certificates received to the total mortality.
		Medical officers of hospitals.	Private medical practitioners.	Hakims and Vaidyas.	Commissioner of Police.	Coroner of Bombay.	Total.	
1931	25,105	4,213	3,484	10	128	621	8,456	33.68
1932	22,856	4,031	2,963	9	170	831	8,004	35.02
1933	27,171	5,111	3,452	19	135	757	9,474	35.06
1934	27,368	5,338	4,174	16	179	762	10,469	38.25
1935	29,287	6,134	3,468	25	218	835	11,280	38.51
1936	29,931	6,286	3,636	9	200	940	11,071	36.98
1937	30,768	6,478	4,329	18	181	924	11,930	38.77
1938	35,999	7,698	4,586	39	219	1,076	13,618	37.83
1939	30,520	7,105	3,887	6	166	1,025	12,189	39.94
1940	29,100	7,036	3,772	12	117	1,179	12,116	41.60

It would be safe, therefore, to assume that the number of deaths due to cancer would probably be in the same proportion in the uncertified group, as in the certified. The total deaths due to cancer in Bombay would, therefore, probably be 2.66 times the number reported in official figures. Table III gives the estimated population for the city of Bombay (Census of India, 1941), the total cancer deaths during the ten years 1931-40 as given in the Administration Reports of the Municipal Commissioner, and the age specific death rate.

TABLE III.

Bombay city estimated population and cancer deaths (all forms, all sites).

Age group.	ESTIMATED POPULATION (1931-40).		TOTAL CANCER DEATHS (1931-40).		AGE SPECIFIC DEATH RATE.	
	Males.	Females.	Males.	Females.	Males.	Females.
0-19	256,665	205,036	46	28	1.8	1.4
20-29	247,133	129,421	75	49	3.0	3.8
30-39	208,625	77,893	201	144	9.6	18.5
40-49	88,089	37,247	464	256	52.7	68.7
50-59	31,231	19,928	485	259	155.3	130.0
60-69	9,000	7,750	250	175	277.7	225.8
70 and over	3,773	3,335	157	109	416.2	326.9

Table IV and Figs. 1 and 2 show the mean annual mortality from cancer per 100,000 living persons arranged according to sex and age groups.

TABLE IV.

Cancer (all forms, all sites) mean annual mortality per 100,000 living at different age groups.

Age group.	New York.	London.	Age group.	Bombay.	Bombay (corrected).
Males.					
0-24	4.4	3.9	0-19	1.8	4.8
25-34	12.8	14.8	20-29	3.0	8.1
35-44	55.8	51.3	30-39	9.6	25.6
45-54	232.7	203.0	40-49	52.7	140.4
55-64	639.4	536.1	50-59	155.3	413.4
65-74	1,216.9	937.1	60-69	277.7	739.6
75 and over	2,051.4	1,102.1	70 and over	416.2	1,108.1

TABLE IV—concl'd.

Age group.	New York.	London.	Age group.	Bombay.	Bombay (corrected).
Females.					
0—24	3·8	3·0	0—19	1·4	3·6
25—34	23·5	17·3	20—29	3·8	10·1
35—44	97·8	84·3	30—39	18·5	49·2
45—54	277·9	241·9	40—49	68·7	183·0
55—64	577·1	453·2	50—59	130·0	346·0
65—74	980·0	730·6	60—69	225·8	601·2
75 and over	1,669·7	981·4	70 and over	326·9	870·4

Mean annual cancer mortality per 100,000
living at each age group.

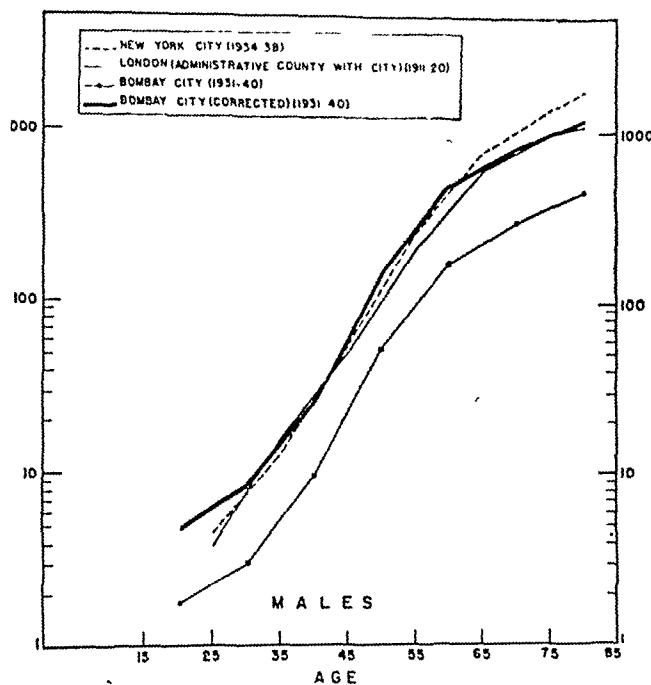


Fig. 1.

Mean annual cancer mortality per 100,000
living at each age group.

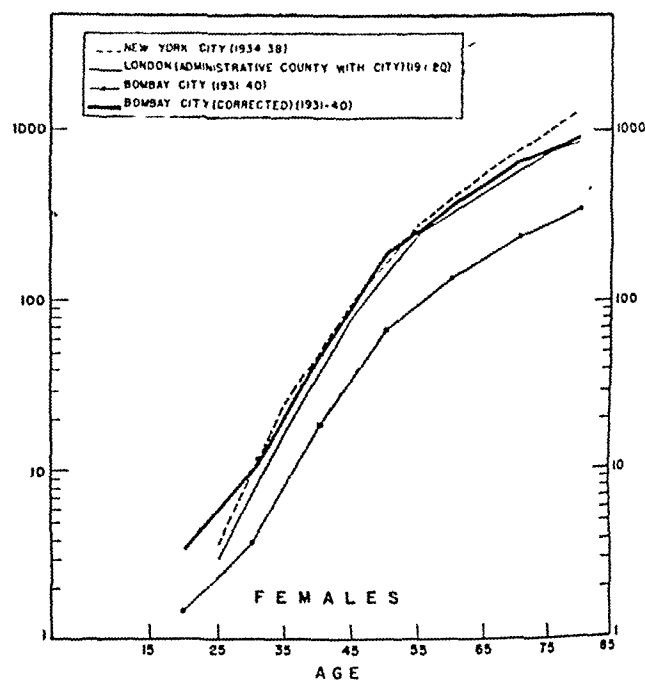


Fig. 2.

It is remarkable that the number of cancer deaths in Bombay city modified as above closely approximates those in London and in New York (Duffield and di Mario, 1941; Registrar-General of England and Wales, 1921). An objection might be raised that the number of cancer deaths in Bombay city would be disproportionately high because of the influx of many people suffering from malignant disease who gravitate from long distances towards the city in search of efficient treatment. This is a valid objection, but our experience shows that, although many people come into the city for treatment, the majority prefer to go back to their home towns to die, when they have been told that the chances of a cure are meagre. Fig. 3 also shows that the population of the city is disproportionately high in

the age groups 20 to 40 because of the large inflow of able-bodied men for employment in many of its industries and that there is a big exodus to their native places of men over 40, when cancer begins to occupy a prominent place in the list of causes of death.

TABLE V.

Age in years :—		1-10.	11-20.	21-30.	31-40.	41-50.	51-60.
Male	{	99,186	118,742	216,827	195,820	75,708	23,553
		2,885	483	692	842	1,930	5,224
Female	{	81,847	82,612	115,293	68,606	31,894	16,937
		3,653	877	1,397	1,560	2,030	3,555

Persons living in Bombay city (1931) and mean annual mortality per 100,000 living in each age group (1931-40).

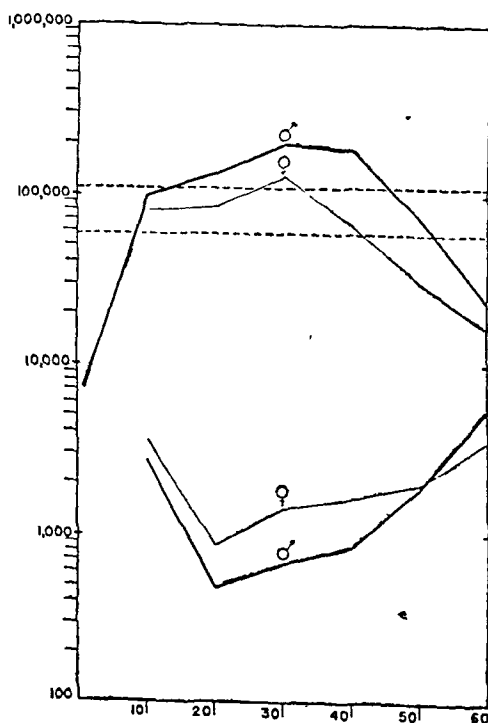


Fig. 3.

An average intelligent person is more interested in knowing his own chances of escaping or dying from cancer, than in the mean annual mortality from it. Table VI and Fig. 4 show the probability of eventually dying from the two diseases, cancer and tuberculosis in Bombay city and U. S. A.

TABLE VI.

Probability of eventually dying of cancer and tuberculosis.

Sex and disease.		AGE IN YEARS.								
		10.	20.	30.	40.	50.	60.	70.	80.	90.
MALES :										
Cancer	...	Bombay	0.0529	0.0546	0.0661	0.0781	0.0741	0.0561
	...	U. S. A.	0.0865	0.0887	0.0922	0.0961	0.0991	0.0969	0.0823	0.0570
Tuberculosis	...	Bombay	0.2825	0.2845	0.2488	0.1779	0.1046	0.0530
	...	U. S. A.	0.0642	0.0621	0.0518	0.0418	0.0317	0.0223	0.0133	0.0066
FEMALES :										
Cancer	...	Bombay	0.0419	0.0465	0.0617	0.0710	0.0631	0.0433
	...	U. S. A.	0.1196	0.1223	0.1271	0.1297	0.1255	0.1126	0.0903	0.0617
Tuberculosis	...	Bombay	0.3560	0.3323	0.2159	0.1078	0.0499	0.0248
	...	U. S. A.	0.0570	0.0516	0.0381	0.0289	0.0222	0.0171	0.0119	0.0058

This table is calculated from the data published for the United States (Registration States), 1924; from Dublin L.I. S.G.O., 1927, Supplement 2, page 279 (10); and for Bombay city (1931-40), computed from the Adm. Reps. Mun. Commr., City of Bombay, 2, 1931-40 (1).

*Probability of eventually dying of tuberculosis
or cancer.*

Bombay city

U. S. A.

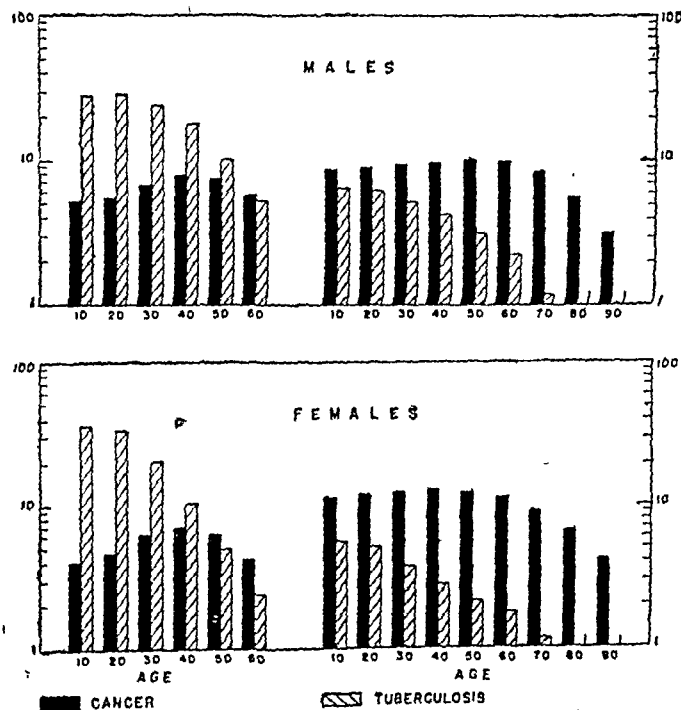


Fig. 4.

The differences in the death rates between the two localities emphasize the importance of examining the age composition of a population when dealing with frequency of deaths due to a disease like cancer. A difference in the age composition of different localities may explain to a certain extent the 4·5 per cent cancer cases in our autopsies as against 13 to 15 per cent in European figures.

It is worthwhile looking little more closely at this question of age composition of our population. The Statistical Year Book of the League of Nations (1939-40) gives interesting life tables about different countries. It will be seen from Table VII, which is based on the information contained in that book, that out of every 100,000 males born in India only 29,000 live to the age of 45 and out of 100,000 females only 26,000. The corresponding figures for England and Wales and for the U. S. A. are 78,000 and 82,000, respectively.

TABLE VII.
Life table : Number surviving out of 100,000 born.

Age.	INDIA (1931).		ENGLAND AND WALES (1930-32).		U. S. A. (1929-31).	
	Males.	Females.	Males.	Females.	Males.	Females.
1	75,126	76,766	92,814	94,545	93,768	95,037
5	60,161	62,817	90,069	92,024	91,738	93,216
10	56,467	59,369	89,023	91,082	90,810	92,466
15	54,112	56,757	88,360	90,420	90,074	91,894
20	51,203	52,833	87,245	89,383	88,904	90,939
25	47,787	47,932	85,824	88,133	87,371	89,524
30	43,931	42,675	84,416	86,792	85,707	87,972
35	39,461	37,266	82,885	85,353	83,812	86,248
40	34,563	31,778	80,935	83,690	81,457	84,256
45	29,439	26,409	78,357	81,660	78,345	81,780
50	24,348	21,464	74,794	78,958	74,288	78,572
55	19,476	17,065	70,041	75,290	68,981	74,321
60	14,933	13,210	63,620	70,204	61,933	68,462
65	10,773	9,761	54,899	63,046	52,964	60,499
70	7,036	6,627	43,361	53,144	41,880	49,932
75	3,848	3,841	29,665	40,040	29,471	37,024

This information is graphically depicted in Figs. 5 and 6. The figures show clearly that roughly a quarter of the infants born in India die during the first year ; and this fact has riveted the public attention, mainly due to the excellent propaganda by infant welfare organizations. It would appear, however, that the problem is not viewed in its true perspective, by emphasizing infant mortality and neglecting the mortality in the older age groups. This situation was recently described by Hill (1944) in the House of Commons. 'The average new-born child in India has an even chance of living to 22 ; in Britain and America the same child has an even

chance of living to nearly 70. This is not, as is commonly suggested, solely a matter of a high infantile death rate; it is due to a mortality which is four to eight times higher than ours right up to the age of 55'. Considered from the point of view of the country this is a serious loss, because it implies a forfeiture of nearly half its population during the most productive period of life. These are the people for whom the family, the community and the state make the biggest sacrifices and who in other countries live long enough to prove of inestimable value by attaining the full span of their usefulness. It is the age group which supplies in other countries men who rule over the destinies of institutions, armies and empires.

Comparison of survivors at different ages per 100 persons born.

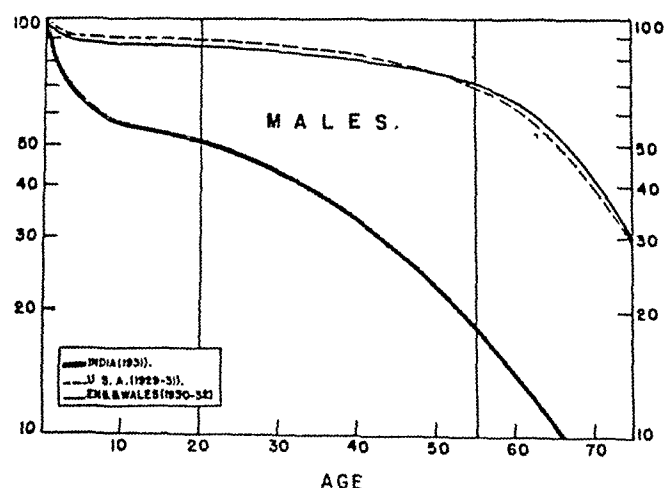


Fig. 5.

Comparison of survivors at different ages per 100 persons born.

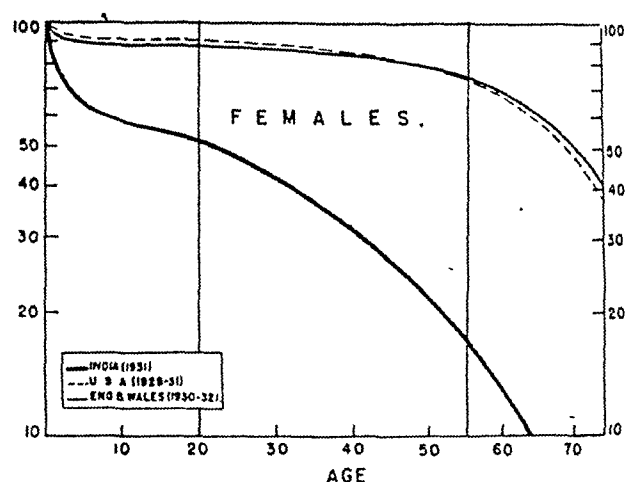


Fig. 6.

It may be permissible to digress slightly from the subject and look into the causes of death which strike the young men and women at the period of their greatest effectiveness. Table VIII shows some of the principal causes of death which were just as common in London and New York hardly 100 years ago as they are in Calcutta, Madras and Bombay to-day. It is probable that the death rate for England and Wales in 1746 was about 35 per 1,000 of the population. It varied from under 30 in some of the healthy county parishes to about 50 for the city of London. It has been stated that of all the children born in London almost three-fourths died before reaching the age of five years. Already towards the end of the 18th century the proportion of children dying in the first five years in London had fallen to about 40 per cent of the children born during that period (Cole and Postgate, 1938). It would be seen that the death rate per 1,000 of the population as well as the infantile mortality in England about the year 1800 closely approximates the figures obtaining in British India now.

TABLE VIII.

Cause—specific death rates per 100,000.*

			Calcutta.	Madras.	Bombay.	New York.	London.	Puerto Rico.
Tuberculosis	270.0	113.0	170.0	47.0	87.0	266.5
Dysentery and diarrhoea	250.0	436.0	252.0	0.0	0.0	415.0
Typhoid	90.0	16.0	40.0	0.2	0.4†	...
Cholera	50.0	0.0	0.0	0.0	0.0	...

* This table is based on data given by Grant (1943) and Villard (1944).

† 1937.

The chief diseases of London during last century according to a book written by Bateman (1819) were 'malignant intermittent and remittent fevers', and just as we find in India to-day (Fig. 7) diseases now known as malaria, typhus (goal fever), typhoid (enteric fever) and relapsing fever were hidden in the list of malignant fevers. Most of these diseases are now known to be associated with dirt, starvation, improper drainage and bad water-supply. Whether the dying out of these fevers in England and America in such a short time could be attributed entirely to draining of marshes, reclaiming of land and improving the nutritional standards of the people or to some other reasons, it has to be accepted that the sequence of events looks temptingly like cause and effect.

Comparison of principal causes of death.

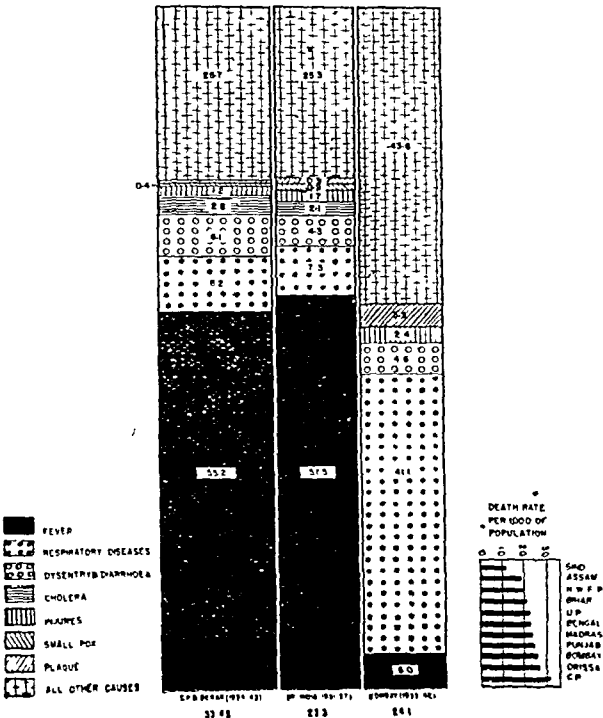


Fig. 7.

Course of mortality from cancer in the death registration states, U. S. A. (1900-30).

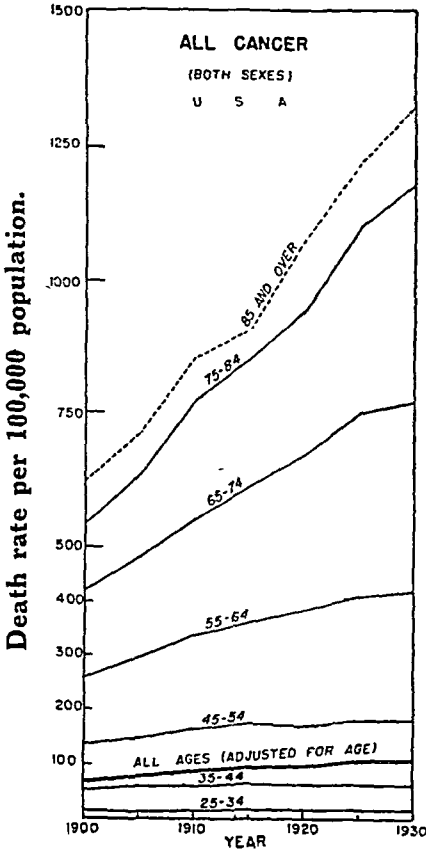


Fig. 8.

The proximity to a nation with a very high standard of living or a healthy climate does not shield a people from the ravages of disease, following in the wake of intense economic exploitation. This is clearly seen in the information available from the American colony of Puerto Rico. A death rate from diarrhoea and enteritis of 415.5, from tuberculosis of 266.5 and from pneumonia of 176.1 per 100,000 of the population, as against the corresponding figures of 14.2, 44.7 and 67.0 for U. S. A. are quite as instructive as the fact that the second largest cause of death in young women between the ages of 15 and 19, and men between 26 and 34, is suicide (Villard, *loc. cit.*). It would also be seen from Fig. 7 that epidemic diseases receive

an undue prominence in our minds. This is probably so because a sudden attack of pestilence causes a greater stir among the health authorities and the people, than does the toll levied continuously by insidious disease.

As regards cancer it is now well known that it occurs most often in people more or less advanced in years and that the mortality from it rises as the number of elderly people in a community increases (Fig. 8). 'So it was in Vienna where between 1923 and 1933 the number of inhabitants over 60 years of age was augmented by 40 per cent and the cancer death rate by 33 per cent' (Oberling, 1944). This may mean that if as an outcome of the several plans, committees and conferences which are meeting everywhere, we eventually succeed in eliminating most of the avoidable disease in India as others have done in their countries, the age composition of our population will necessarily change and we shall have to make more extensive arrangements for the investigation and treatment of cancer. It is likely that we may be confronted with a new situation, so that with every reduction in the mortality in the earlier age groups by an improvement in the conditions of life, a larger number of persons may be exposed to the menace of malignant disease.

The facts mentioned so far suggest that the total incidence of cancer in India, Europe and America would show little difference if sufficiently accurate statistics were available and that any differences that may be apparent would disappear with a greater uniformity of age composition of the populations. This should not be construed necessarily to mean that there are no racial differences regarding cancer in the human species or that cancer is a constant function of the age of the population. Even though it may be shown that the incidence of total cancer is approximately the same in most races, it is certain that the incidence of separate forms of cancer or rather of various parts of the body is very different in different people. The higher frequency of skin cancer among the fair Nordic peoples, and the greater incidence of cancer of the body of the uterus in European Jews has been commented upon by European observers (Schottky, 1937). The high incidence of ovarian tumours and uterine fibroids in women, and breast cancer in men, as well as the low incidence of cancer of the exposed skin in American negroes has also been noted in several recent publications (Schrek, 1944). A difference in incidence of cancer according to site is also shown in Fig. 9. It will be noticed

Annual death rate per 100,000 living of each sex.

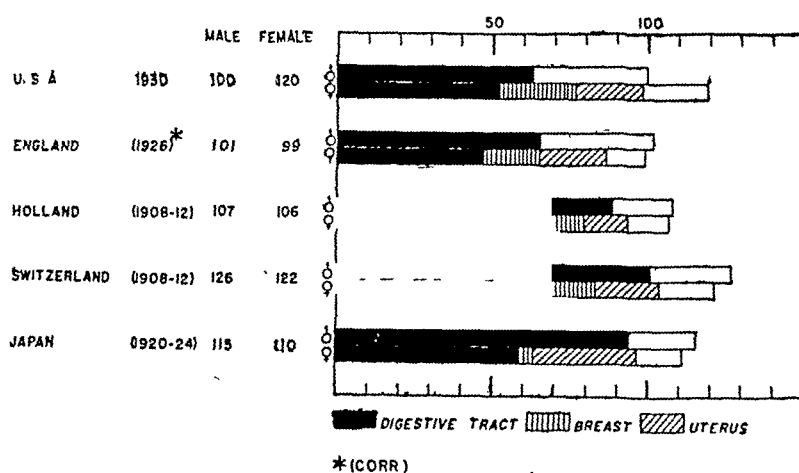


Fig. 9.

that the total incidence of cancer in the five countries and the two sexes is very nearly the same. The differences, however, between cancer in those organs which are specific to the two sexes and in the deeper viscera between the five people are quite noticeable. Our own

observations (Khanolkar, 1944) regarding the incidence of oral cancer have revealed some interesting differences which may be briefly referred to below :—

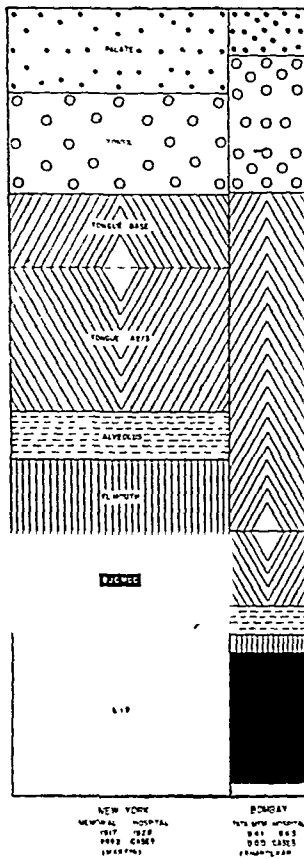


Fig. 10.

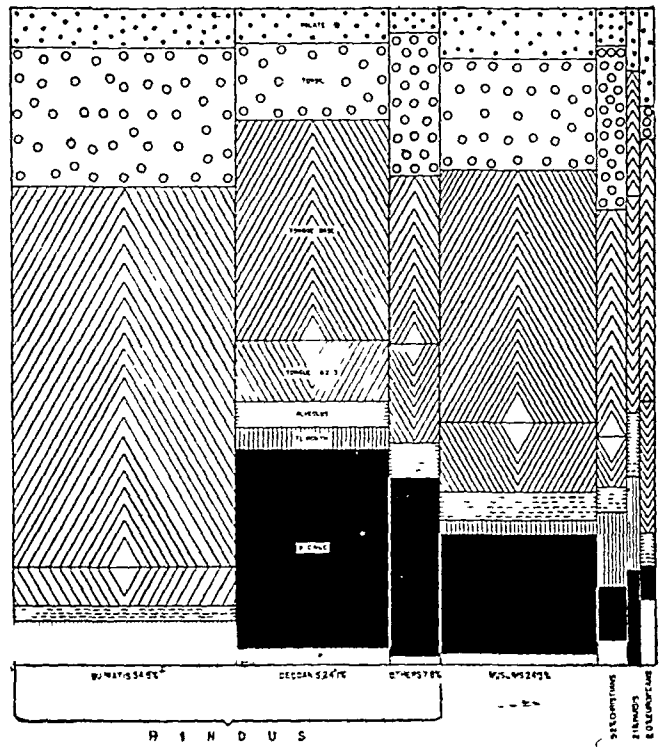


Fig. 11.

1. Cancer of the lip accounts for nearly 20 per cent of the oral cancer at the Memorial Hospital in New York and only 1·7 per cent at the Tata Memorial Hospital in Bombay. The cancer mortality figures for U. S. A. show that the high figures for this type of cancer are not due to an unusual attendance at the Memorial Hospital of those suffering from lip cancer.

2. In New York cancer of the anterior two-thirds of the tongue is twice as common as at the base, whereas in Bombay these proportions are reversed.

3. As compared to Gujaratis, cancer of the buccal mucosa is six times (5·2 against 30·8 per cent) more common among Deccanis, while that of the tongue is one and a half times as common among Gujaratis (64·3 against 42·8 per cent), the major portion of this higher incidence being in the posterior third of the tongue.

Men and women differ significantly as regards the incidence of cancer of the mouth in all countries. This difference becomes all the more striking when the frequency of cancer in the two sexes is considered according to the different parts of the mouth cavity. The striking difference in the two sexes in Gujaratis as regards cancer of the base of the tongue and the adjoining portions of the larynx raised some doubt regarding an uneven attendance of males and females at the Tata Memorial Hospital. It was felt that Gujarati women may be avoiding attendance at our clinics even when suffering from mouth cancer (Table IX). Through the kindness of some of our past students who are at present on the visiting staff of a hospital where the patients are predominantly Gujarati Hindus, we are in a position to present the following data (Table X).

TABLE IX.
Tata Memorial Hospital.
Cases 1-5,000 (1941-43).

				M.	F.
Total carcinoma	2,021	991
Total malignant disease	2,205	1,058
Benign tumours	193	187

				TOTAL.		DECCANIS.		GUJARATIS.	
						M.	F.	M.	F.
Oral cancer	1,041	203	57	336	17		
Cheek	170	51	25	13	6		
Antr. tongue	101	19	6	18	4		
Base tongue	439	73	13	203	2		
Larynx	323	96	2	99	5		
Œsophagus	102	32	8	28	1		
Breast	164	2	38	...	6		
Cervix	293	...	148	...	45		

TABLE X.
Gujarati Hindus.

				S. H. H., 1934-43.		T. M. H., 1941-43.	
				Total cancer.	Tongue cancer.	Total cancer.	Tongue cancer.
Males	349	135 (38.68 per cent)	555	217 (39.09 per cent)	
Females	105	8 (7.63 „ „)	108	5 (4.63 „ „)	
Not stated	19	2	
Ratio M : F	3.22 : 1	16.87 : 1	5.13 : 1	43.4 : 1	

It will be seen that proportion of Gujarati males to females suffering from all types of cancer at that hospital (S. H. H.) during the last ten years was 3 : 1, at the Tata Memorial Hospital the proportion was 5 : 1. In the case of tongue carcinoma the proportions were 17 : 1 and 43 : 1. This difference between the two sexes is therefore not accidental and deserves careful study.

Khanolkar and Suryabai (1946, in press) have recently studied the frequency of cancer of the palate in Andhra, which is probably associated with the peculiar method of smoking a locally made cigar. This was first suggested by Kini and Rao (1937) as a result of an analysis of his cases in Vizagapatam.

Primary cancer of the liver is a rare disease in Europeans. Among the Bantu labourers in the Witwatersrand Gold Mines in South Africa it accounted for 229 out of 253 or 90·5 per cent of all cases of cancer (Kinnaway, 1944). It has also been described as the commonest type of cancer in the Malays and Chinese of Batavia, the Chinese of Singapore, the Filipinos in Manila and the inhabitants of Sumatra (Degorce, 1913 ; Snijders and Straub, 1923). The condition has been mentioned as relatively common in Madras by Basu and Vasudevan (1929). The high incidence of this type of cancer is not purely racial in character and it is likely that it is probably attributable to the economic backwardness of all these people (Gilbert and Gillman, 1944). It affords an interesting example of the results of modern exploitation on large masses, who subsist in a state of continued penury and malnutrition on an income of about Rs. 200 to Rs. 500 per annum. A monotonous diet, poor in proteins, with a low content of certain amino-acids probably results in damage to the liver (Glynn and Himsworth, 1944) leading to a cancerous proliferation of its cells.

In conclusion, it might be stated that the welfare of each individual is determined by his inheritance, by his environment, by the acts he performs and the habits which he acquires during the course of his life. The importance of hereditary or racial factors is probably different for each type of cancer and in any case not so impressive as the other two factors. The clinical experience of trained observers, as well as a guarded use of official statistics, suggests that so far as the big cities of India are concerned and where alone a sufficient number of doctors are available and the equipment for diagnosis and registration is operative, the population is as susceptible to cancer in general as the people in Europe and America. This opinion is not accepted by medical men who have an incomplete understanding of the problem and have been led away by clinical impressions based on insufficient observations ; and also by statisticians who 'have approached the subject without informing themselves of the objective value of the data upon which they relied' (Bashford, 1905). It is hoped that the observations presented here will dispel the confusion caused by most of the contributions to this subject and that it may reasonably be asserted that the conviction of those who maintain 'that cancer in its different forms is unquestionably relatively very rare throughout India' (Hoffman, *loc. cit.*) is erroneous. Numerous letters and articles encumber reputed medical journals, and describe the rarity of cancer in Africans, Egyptians, Chinese, Arabs and Indians and impress medical practitioners with their appearance of authority. It is not unusual therefore to come across many unfortunate individuals in India who have been treated by their medical advisers for everything else but cancer, until the disease has advanced beyond all hope of effective treatment, because the doctor was convinced that his patient could not have been suffering from such a rare condition. It has been necessary to obtain a reasonably accurate idea of the prevalence of cancer in this country for another reason. Arrangements and organization for medical relief should be based on the relative frequency of different diseases. If cancer is really rare, the present deplorable lack of specially trained men to combat the disease would be justifiable ; on the other hand, if it is much more prevalent than it is generally believed to be, effective measures should be immediately adopted to improve the diagnostic and treatment centres and to revise the attitude of the medical and public health professions, as well as of the lay public, towards malignant disease.

SUMMARY AND CONCLUSIONS.

1. A study of the clinical material from medical institutions in India, as well as a guarded use of official statistics suggests that Indians are as liable to suffer from cancer as the inhabitants of Western countries.
2. Evidence has been presented to show that though the total incidence of cancer in several countries may show small differences, the incidence of cancer in various parts of the body is markedly different in different peoples.
3. The implications arising out of these findings are briefly discussed.

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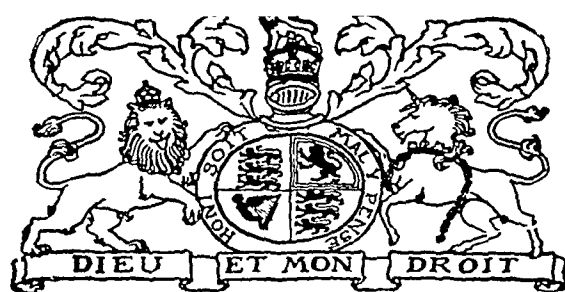
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THE PREPARATION OF AN ANTIGEN FROM THE KEDROWSKY'S BACILLUS FOR THE COMPLEMENT-FIXATION TEST FOR KALA-AZAR.

BY

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INTRODUCTION.

THE W.K.K. (Witebsky, Klingenstein and Kuhn) antigen prepared from the tubercle bacillus has been successfully used for a complement-fixation test in the diagnosis of kala-azar. This antigen was originally prepared for use in the complement-fixation test in the diagnosis of tuberculosis, but the results were not satisfactory. A South American worker reported positive reactions in 70 per cent cases of leishmaniasis. Later, Lowe and Greval (1939) confirmed these results in Indian kala-azar. Sen Gupta (1943) reported positive results in 97 per cent of a large series of cases of kala-azar.

Dharmendra and Bose (1941) prepared antigens by the W.K.K. method from a number of acid-fast bacilli, including the tubercle bacillus, and the so-called leprosy bacilli of Duval, Bayon, Lleras, and Kedrowsky. They reported that sera from cases of kala-azar and severe cases of leprosy fixed complement in the presence of these antigens, and that all the antigens behaved in a similar way.

Because of the ease with which some of the acid-fast bacilli, other than the tubercle bacillus, grow in cultures, it was decided to try, in the complement-fixation test for kala-azar, an antigen prepared from one of them in place of the original W.K.K. antigen prepared from the tubercle bacillus. For this purpose, the antigen from the Kedrowsky's bacillus, as prepared in the Leprosy Department of the School of Tropical Medicine, Calcutta, was selected for trial, and has been found to be very satisfactory. Using this antigen, Sen Gupta (1944) reported positive reactions in 93 per cent cases of kala-azar; and negative reactions in 99 per cent of all cases likely to be considered in the differential diagnosis of kala-azar; in the remaining 1 per cent of these cases the reaction was doubtful. The antigen has, therefore, proved to be of great value in the diagnosis of kala-azar by complement fixation.

Because of the usefulness of this antigen prepared from the Kedrowsky's bacillus, several inquiries have been received regarding the method of its preparation. In this article, therefore, it is proposed to describe the method of preparation of the antigen in detail.

THE METHOD OF PREPARATION OF THE ANTIGEN.

The antigen consists essentially of the acetone-insoluble fraction of the pyridine extract of the alcohol-insoluble portion of the bacilli. For use it is dissolved in benzol, and lecithin is added to make it more sensitive. The details of the method are described below:—

(1) Kedrowsky's acid-fast bacillus is grown on glycerine broth in flasks, each containing about 200 c.c. of the medium, the pH of the medium being adjusted to 7.3 to 7.4. In about three weeks' time, there is a luxuriant growth of the bacillus on the surface of the medium.

(2) The bacillary growth is collected from the medium by filtration through ordinary filter-paper; it is washed free from traces of the medium with three changes of sterile distilled water, and then twice with rectified spirit (96 per cent alcohol). The washed residue is transferred to a desiccator, and dried *in vacuo*.

(3) The dried bacillary mass is extracted with 90 per cent alcohol in a flask fitted with a reflux condenser for three hours over a water-bath, and is then kept overnight in a refrigerator. The residue is then filtered free from alcohol and again dried *in vacuo*.

(4) This alcohol-insoluble portion of the bacilli is extracted with pyridine for eight hours at 135°C. to 140°C. in a Soxhlet apparatus over glycerine-bath.

(5) The pyridine extract is transferred to an evaporating dish, which is placed over a hot-air bath at 70°C. and the extract evaporated to dryness, taking care to avoid any charring. The residue is the pyridine-soluble fraction of the alcohol-insoluble portion of the bacilli.

(6) This pyridine-soluble fraction of the alcohol-insoluble portion of the bacilli is next extracted with acetone in a Soxhlet apparatus for two hours over a water-bath. The fraction insoluble in acetone is dried, and is used as the antigen.*

The dried antigen can be stored for future use in sealed ampoules, in a refrigerator.

(7) For actual use a weighed amount of the dried antigen is dissolved in benzol, and is sensitized by the addition of lecithin. This is done as under :—

0.1 g. of the antigen is dissolved in 10 c.c. of benzol by trituration.

Five c.c. of 1 per cent alcoholic solution of lecithin is evaporated to dryness, and the residue is taken up in the 10 c.c. of the benzolic solution of the antigen, which is then filtered through Whatman filter-paper, any loss of benzol being made up by addition of fresh benzol. This is the lecithinized benzolic solution of the antigen which is used for the test.

(8) The prepared antigen is kept in an incubator at 37°C. for 10 to 15 days before it is used. At the time of use the antigen is titrated for (a) anti-complementary activity, (b) hæmolytic property, and (c) specific complement fixation, according to the method described by Sen Gupta (1945).

SUMMARY.

A description is given of the technique used for obtaining the antigen from the Kedrowsky's bacillus. This method is applicable for the preparation of similar antigens from other acid-fast bacilli. In the case of the tubercle bacillus, the culture should first be autoclaved to kill the bacilli.

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* An idea of the yield of the different fractions, and finally the dried antigen, will be obtained from the following data from one experiment :—

- (1) 21.7 g. of dried bacillary mass was obtained from the growth in 20 flasks, each containing about 200 c.c. of glycerine broth.
- (2) The alcohol-insoluble portion of these bacilli weighed about 19.6 g.
- (3) The pyridine-soluble fraction of the alcohol-insoluble portion of the bacilli weighed about 3.5 g.
- (4) The acetone-insoluble portion of the above fraction, which is the dried antigen, weighed about 2.3 g. This is about 1/10th of the weight of the dried bacillary mass.

VITAMIN A AND CAROTENE CONTENT OF GHEE (CLARIFIED BUTTER) AND 'FORTIFIED' MARGARINE.

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GHEE is clarified butter-fat prepared from the milk of the cow and the buffalo. It is the principal form in which milk-fat is consumed in India. It has been estimated that 58 per cent of the total production of milk, amounting to 13·17 million tons, is used in the manufacture of ghee (Report on the Marketing of Milk in India and Burma, 1941). Even in warm climates, ghee can be kept without deterioration for considerable periods of time, and one sample is reported to have remained in good condition after two years of storage. The ghee consumed in India is mostly made from buffalo milk. The latter has a higher fat content, and buffalo ghee is said to have better keeping qualities than cow ghee. Further, the buffalo ghee is white in colour, is hard and has a well-defined structure as compared with the cow ghee which has a yellowish colour and a softer consistency, and consequently the former fetches a better price.

Davies (1940) describes the method of manufacture as follows: 'When ghee is made in small quantities, milk is allowed to sour (ripen) at a fairly uniform temperature until clotted, or otherwise has been changed into *dahi*. A liquid (or solid) fat-rich fraction is then churned from the *dahi* by a rotating paddle working in the *dahi* contained in an earthenware jar (*ghara*). The fat is collected from the paddle and the surface of the *lassi*, and transferred, usually by hand, into another earthenware or brass vessel, in which the daily yields of butter are collected until there is sufficient to merit the separation of *kutcha* ghee by boiling. The butter is melted and the underlying layer of water and curd brought to the boil, while the fat collects on top. The water is boiled off briskly at first and then more gradually. A scum of solid material forming on top of the fat is skimmed off carefully from time to time. When all the water has been evaporated the temperature of the fat rises above 100°C. appreciably and the curd chars to a brown colour and particles are seen in the convection currents in the fat. The butter has then been sufficiently boiled, and is allowed to cool somewhat while the curd particles settle. The fat layer is then carefully removed into suitable vessels as free from solid curd as possible and allowed to cool and crystallize. The hot ghee may or may not be filtered through muslin into the containers.'

The *per capita* consumption of ghee in India is in general low. Megaw (1933) estimated average consumption of ghee on the part of adults to be 0·32 oz. Diet surveys carried out in various parts of the country and among different classes of people (Aykroyd, 1939) showed that the *per capita* consumption ranged from almost *nil* to 2·0 ounces, the latter being among business and professional families with an income of Rs. 100 to Rs. 1,000 per month. Dr. K. Mitra, Nutrition Officer, Bihar, in a survey of 2,566 families in that province, found that the consumption varied from almost *nil* to 1·5 ounces per consumption unit per diem (personal communication). He further observes that 'ghee is mainly used for cooking purposes and very rarely consumed raw'. Dr. Musa Khan, Nutrition Officer, Public Health Department, Punjab, in a diet survey of agricultural families in the Lyallpur District, found that 'ghee was used in two ways: About one-third was used for smearing *chapattis* or taken with bread after being sweetened with sugar . . . The rest, about two-thirds, was used for frying purposes' (personal communication). Such a low consumption is not surprising as the total available supplies are small in relation to the size of the population. The total quantity produced hardly suffices

for a *per capita* consumption of 0.2 oz. daily. Some trade existed in ghee in pre-war times, but it was not of much importance as the imports nearly equalled the exports, the figures amounting to a little over 2,500 tons. Because of the short supply of ghee the manufacture of vegetable ghee (Vanaspati) is assuming considerable importance. Vegetable ghee, being cheaper than animal ghee, is being increasingly used by certain sections of the Indian population, and for the same reason the former is widely used for adulterating genuine ghee.

The vitamin A activity of milk-fat is due to the presence of vitamin A, and of carotene when present. Butter has been assayed for vitamin A activity by a number of workers (Crawford, Perry and Zilva, 1932; Baumann and Steenbock, 1933; Shrewsbury and Kraybill, 1933; Booth, Kon, Dann and Moore, 1933; Baumann, Steenbock, Beeson and Rupel, 1934; Berl and Peterson, 1943). Sherman and Smith (1931) state that 'butter appears usually to contain about 30 to 50 units of vitamin A per gramme'. Berl and Peterson (*loc. cit.*), working in the U.S.A., found that March butters had a vitamin A potency of approximately 21 International Units per gramme, while July and September samples contained 40 International Units per gramme. The vitamin A activity of ghee has been determined by a number of workers in India (Banerjee, 1936; De and Majumdar, 1938; Grewal and Kochhar, 1938; Majumdar, 1941; Muthanna and Seshan, 1941). Grewal and Kochhar (*loc. cit.*) reported the following values (International Units per g.): ghee prepared in the laboratory, 30.7; ghee prepared from summer butters, 19.0; from winter butters, 27.6; samples collected from villages round Lahore, 12.2; in Lahore, 16.4. Muthanna and Seshan (*loc. cit.*), working with samples of ghee received from Sind and Bengal, reported average values of 12 μ g. (36 I. U.) and 8 μ g. (24 I. U.) per gramme respectively. De and Majumdar (*loc. cit.*) obtained an average value of 7.8 μ g. per g. for ghee.

The relative nutritive value of animal ghee and vegetable ghee is a controversial question of considerable importance. Detailed information about the amount of vitamin A actually obtained by the consumer from animal ghee is necessary in order that the advantage of consuming animal rather than vegetable ghee, in respect of vitamin A intake, can be assessed. In the present investigation bazaar samples from various parts of the country have been assayed, together with pure samples prepared from good milk under optimum conditions. The effect of heat on vitamin A in ghee has been investigated. Experiments on effect of heating on the carotene present in 'fortified' Margarine are also reported.

METHODS.

Vitamin A was estimated tintometrically on the non-saponifiable fraction by means of the Carr-Price reaction. Experiments with the spectrograph gave a figure of 53 for the conversion of the Carr-Price value to vitamin A in I. U. per g. Carotene was estimated in the non-saponifiable fraction by dissolving it in petroleum ether; the colour was matched in terms of yellow units in a Lovibond tintometer and the carotene estimated by using a standard curve plotted with pure β -carotene. During the various stages of the experiment, from the saponification to the final stage, the experiments were conducted in an atmosphere of nitrogen and in a partially dark room where there was a minimum of diffused light.

The estimation of carotene in 'fortified' Margarine was carried out in a similar manner on the non-saponifiable fraction. No attempt was made to eliminate other interfering pigments as they were not found to be present in the carotene concentrates used for the fortification of the hydrogenated fat.

RESULTS.

Tables I to VI show the results of carotene and vitamin A assay of various samples of cow and buffalo ghee manufactured under different conditions.

TABLE I.

Ghee obtained from fresh milk of well-fed cross-bred cows at the Dairy Farm of the Government Agricultural College, Coimbatore.

Serial number.	Carotene in I. U. per g.	Vitamin A in I. U. per g.	Total vitamin A activity in I. U. per g.
1	11.7	25.1	36.8
2	9.6	23.8	33.4
3	17.5	24.2	41.7
4	14.6	23.3	37.9
5	6.5	21.6	28.1
6	8.5	32.8	41.3
AVERAGE	11.4	25.1	36.5

TABLE II.

Samples of bazaar ghee sold in various places of the Madras Presidency and certified to be unadulterated by the Public Health Analyst.

Serial number.	Place.	Reichert value.*	Vitamin A in I. U. per g.
1	Ramachandrapuram ...	25.8	6.1
2	Tirumalai Hills ...	28.2	6.9
3	Ongole ...	33.3	14.7
4	Jammalamadugu ...	28.6	5.4
5	Sembiam ...	28.6	7.1
6	Parvathipuram ...	34.1	7.5
7	Pulivendla ...	26.8	8.2
8	Ootacamund ...	25.7	4.6
9	Ponnur ...	29.3	9.1
10	Samalkot ...	28.8	8.9
11	Ootacamund ...	26.6	7.0
12	Masulipatam ...	30.3	13.8
13	Chittoor ...	28.4	10.8
14	Bezwada ...	26.2	8.2
AVERAGE			8.5

* Figures supplied by the Public Health Analyst.

TABLE III.

Samples of buffalo ghee received from the Chemist, Public Health Department, Lahore.

Serial number.	Period of lactation.	Diet of buffalo.	Vitamin A in I. U. per g.
1	6 months	Maize green, green jawar stalks, dry paddy stalks, green grass.	18.0
2	6 "	Cotton-seed 4 lb., tara mira (oil-seed) 1 lb., oil-cake 1 lb., maize, green grass.	9.9
3	4 "	Cotton-seed 4 lb., green jawar stalks and green grass.	9.1
4	5½ "	Cotton-seed 1 lb., ground gram 4 lb., green jawar stalks, green grass.	7.8
5	1½ "	Cotton-seed 4 lb., tara mira (oil-seed) 1 lb., gram 2 lb., green jawar stalks, green grass.	8.2
6	7½ "	Cotton-seed 3 lb., green jawar stalks and green grass.	12.3
7	1 "	Gram 4 lb., oilcake 1 lb., dry bran 1 lb., bhoosa and green sugar-cane.	10.8
8	2 "	Gram 4 lb., oilcake 1 lb., millet 4 lb., dried jawar stalks and bhoosa.	6.5
9	2 "	Gram 6 lb., millet 4 lb., oilcake 2 lb. and bhoosa.	Trace.
10	2½ "	Cotton-seed 1½ lb., oilcake 1½ lb., millet 1½ lb. and bhoosa.	5.4
11	10 days	Cotton-seed 4 lb., gram, millet and oilcake each 2 lb., bhoosa and dried jawar stalks.	13.8
12	4 months	Cotton-seed 2 lb., gram 2 lb., oilcake 1 lb., bhoosa and dried jawar stalks.	4.1
AVERAGE ...			8.8

TABLE IV.

Blended ghee samples from the Ghee Heating Centre, Agra.

(Ghee obtained from eight surrounding districts of Agra. The ghee was heated to 75°C., the fire withdrawn and the different samples mixed and blended.)

Sample number.	Vitamin A in I. U. per g.
1	10.4
2	12.1
3	11.7
4	13.6
5	14.3
6	13.2
7	11.2
8	11.9
9	13.8
10	11.7
11	11.5
12	11.5

AVERAGE 12.2

TABLE V.

Buffalo ghee (unadulterated) obtained from villages round Coimbatore.

Serial number.	Source.	Vitamin A in I. U. per g.
1	Coimbatore ...	12.7
2	Velandipalayam ...	17.7
3	Sankanur ...	12.1
4	Karandapalayam ...	11.9
5	Kuppakonaputhur ...	13.4
6	Edayapalayam ...	11.2
7	Pappanaikkanpudur ...	16.0
8	Mallagoundanpalayam ...	10.2
9	Pappanpatti ...	14.5
10	Arukkanpalayam ...	1.4
11	Nellikonanpalayam ...	6.3
12	Karpuranpalayam ...	9.1
13	Singanallur ...	8.2
14	Kalapatti ...	13.6
15	Shouripalayam ...	11.7
16	Kuppakonaputhur ...	8.4
17	Nallampalayam ...	9.5
18	Coimbatore ...	9.3

AVERAGE ... 11.0

TABLE VI.

Miscellaneous samples of ghee obtained from the bazaar in various places in South India.

Serial number.	Source.	Total vitamin A in I. U. per g.
1	Coimbatore ...	Nil
2	Karamadai ...	Nil
3	Perianaikkanpalayam ...	11.0
4	Coonoor ...	21.8*
5	Tirupur ...	17.0*
6	Coonoor ...	13.0
7	Pollachi ...	9.9
8	Tirupur ...	8.8
9	Coimbatore ...	3.2
10	Travancore ...	Nil
11	" ...	2.2
12	" ...	2.1
13	" ...	16.5*
14	" ...	6.9
15	" ...	Nil
16	Satyamangalam... ..	5.2
17	Karamadai ...	6.9
18	Coonoor ...	9.0
19	Coimbatore ...	15.9*
AVERAGE		7.9

* These samples were stated to be cow ghee.

Table VII sets out the results of experiments on the loss of vitamin A activity during the heating of ghee and Margarine 'fortified' with carotene. It should be noted that while ghee has very little moisture, 'fortified' Margarine has the following composition: hydrogenated vegetable fat 83 per cent; common salt (NaCl) 2 per cent; water 15 per cent (i.e. a water content similar to that of butter).

TABLE VII.

Loss of vitamin A activity during the heating of ghee and Margarine 'fortified' with carotene.

Treatment.	VITAMIN A ACTIVITY IN I. U. PER G.	
	Ghee.*	'Fortified' Margarine.†
Original sample	19.1	22.2
Heated for 20 minutes—maximum temperature 130°C. ...	14.8	11.6
∴ Loss of vitamin A activity	22.6 per cent	47.7 per cent
Heated for 20 minutes—maximum temperature 170°C. ...	7.4	3.2
∴ Loss of vitamin A activity	61.6 per cent	85.6 per cent

* Average of two samples.

† Average of three samples.

COOKING EXPERIMENTS WITH GHEE AND 'FORTIFIED' MARGARINE.

Twelve *purees* (fried wheat cakes) were made from a uniform dough consisting of 250 g. of whole-wheat flour (atta), 10 g. of fat (ghee or 'fortified' Margarine), and the requisite quantity of water for kneading into a uniform consistency. These were fried one at a time in excess of fat (ghee or 'fortified' Margarine, 250 g.) in a frying-pan. The temperature during frying was about 170°C. Each *puree* after frying weighed about 25 g. which included about 8 g. of fat absorbed during frying. Vitamin A activity was estimated both in the fat left over after frying and in the fried *purees*. The results are shown in Table VIII:—

TABLE VIII.

Loss in vitamin A activity during frying of purees.

Serial number.	Treatment.	'FORTIFIED' MARGARINE.		GHEE.	
		Vitamin A activity in I. U. per g.	Per cent of original.	Vitamin A activity in I. U. per g.	Per cent of original.
1	Original sample of fat ...	14.0	100	23.0	100
2	Fat heated till moisture was got rid off	3.3	23.6
3	Fat after frying first <i>puree</i> ...	2.9	20.7
4	" " " second " ...	1.1	7.9	13.2	57.4
5	" " " third " ...	1.0	7.1
6	" " " fourth "	8.0	34.8
7	" " " sixth "	4.5	19.6
8	" " " eighth "	<i>Nil or trace</i>	0
9	" " " tenth "	<i>Nil</i>	0
10	First <i>puree</i> ...	1.0	7.1	3.5 (average)	15.2
11	Second " ...	0.8	5.7		
12	Third " ...	0.4	2.9	1.5 (average)	6.5
13	Fourth " ...	0.3	2.1		
14	Fifth and sixth <i>puree</i> —average	0.6	2.6
15	Seventh and eighth <i>puree</i> —average	<i>Nil or trace</i>	0
16	Ninth and tenth <i>puree</i> —average	<i>Nil</i>	0

Six to ten potato chips were fried simultaneously in excess (250 g.) of 'fortified' Margarine and the carotene estimated. The results are given in Table IX.

TABLE IX.

Loss in carotene content of 'fortified' Margarine during frying of potato chips (six to ten chips were fried at a time).

Treatment.		Carotene content in I. U. per g.	Per cent of original.
Original sample	...	20.4	100
Same after first frying	...	7.8	38.2
" " second frying	...	5.1	25.0
" " third frying	...	2.2	10.8
Potato chips of first frying	...	6.6	32.4
" " of second frying	...	2.5	12.3
" " of third frying	...	Nil or trace	0
" " of fourth frying	...	Nil	0

Halwa is a common Indian sweet preparation in which is incorporated fair amounts of ghee. The following is the method commonly employed in its preparation: *suji* (semolina), sugar and ghee are the ingredients used in the proportions of 2: 2: 1. The *suji* is first fried in about one-eighth of its weight of ghee in a frying-pan until it assumes a light brown colour. Water is then added, followed by sugar, and the mixture stirred until cooked into a semi-solid mass. The pan is then removed from the fire and the remaining ghee (three-eighths of the original weight of *suji*) is incorporated into the preparation. The loss in vitamin A activity in the fat of the *halwa* was estimated, the results being given below:—

	Vitamin A in I. U. per g.*
Original sample of ghee	23.0
Fat in <i>halwa</i>	9.8
∴ Loss in vitamin A potency	57.4 per cent

* Average of results of two experiments.

STORAGE TESTS WITH CAROTENE CONCENTRATES AND 'FORTIFIED' MARGARINE.

Carotene concentrates used in the fortification of hydrogenated fats retain unimpaired their vitamin A activity when stored in amber-coloured bottles at ordinary room temperature. Concentrates with a potency of about 75,000 to 80,000 I. U./g. were stored in well-stoppered amber-coloured bottles in an incubator maintained at 37°C. and at room temperature which ranged during the period of experimentation from 18°C. to 23°C. The carotene contents of these samples, estimated after eight months' storage, showed that there was practically no loss in potency.

In the commercial manufacture of 'fortified' Margarine, there is an overall loss of about 20 to 25 per cent of carotene. This loss is due to the cumulative effect of both light and heat. Once the finished product is put in hermetically sealed tins, the vitamin A activity of the sample remains unimpaired for several months. In one typical experiment, two tins of 'fortified' Margarine were selected at random from a batch manufactured on the same day. One was assayed immediately and the other after six months' storage at room temperature. Both gave almost identical values, showing that carotene was not destroyed when the Margarine was stored in hermetically sealed tins.

The opened tin of 'fortified' Margarine was set aside with the contents partially exposed to diffused light in the room. At the end of 3 and 6 months of storage under such conditions, carotene assay was carried out on representative samples. The 'fortified' Margarine was seen to change its colour, the portions exposed to light being bleached while those away from the light took in varying shades of yellow. There was a loss of about 25 per cent of carotene in 3 months and about 42 per cent in 6 months' storage.

To study the effect of slightly higher temperatures—temperatures more likely to prevail in the plains of India—storage tests were conducted at 37°C. in an incubator. Similar tests were carried out on a fluid edible oil 'fortified' with carotene with a view to finding out whether the physical state of the fat medium has any effect on the stability of the carotene contained in it. Ground-nut oil, one of the cheapest edible oils of India and used in blending shark-liver oils, was chosen for the test. A bulk sample of the ground-nut oil was 'fortified' with carotene concentrate in such dilution as to give the same concentration in the final mixture as the 'fortified' Margarine. A number of 1 oz. stoppered glass-bottles were filled with the fortified ground-nut oil and 'fortified' Margarine; six in each group were stored in an incubator at 37°C., while another batch of six was stored in the room away from bright light. Estimations of carotene were made initially and at stated intervals on these samples and the percentage loss of carotene on storage calculated. The results are recorded in Table X:—

TABLE X.
Percentage loss of carotene on storage.

Storage period in weeks.	AT ROOM TEMPERATURE.		AT 37°C. (INCUBATOR).	
	'Fortified' Margarine.	'Fortified' ground-nut oil.	'Fortified' Margarine.	'Fortified' ground-nut oil.
1	0	11.6	16.2	11.6
2	20.5	14.8	23.0	19.0
3	20.5	17.9	27.4	20.3
5	31.0	26.4	34.1	30.8
10	41.7	36.5	40.5	45.0
15	47.3	50.0	54.8	47.0

DISCUSSION.

It will be seen from Tables I to VI that cow ghee contains both carotene and vitamin A, while buffalo ghee is devoid of carotene and contains only vitamin A. The vitamin A activity of ghee made from the milk of well-fed cross-bred cows was found to be on an average 36.5 I. U. per gramme, to which carotene contributed 31.2 per cent. This figure is in contrast

to 15 per cent recorded by Baumann and Steenbock (*loc. cit.*) in the case of butter. The average vitamin A content of bazaar samples of ghee found to be unadulterated by the Public Analyst, Madras, was 8.5 I. U. per g. (range from 4.6 to 14.7, Table II). Samples of buffalo ghee prepared from milk of buffaloes fed with known quantities of concentrates contained on an average only 8.8 I. U. per g. From Table III it will be seen that the relation between feed and vitamin A in ghee is not very marked, but, in general, it may be said that when the feed contains plenty of fresh green fodders, there is observed an increase in the vitamin A content of the ghee. The period of lactation does not seem to be correlated to the vitamin A content of the butter-fat. The average vitamin A content of ghee samples from the Ghee Heating Centre, Agra, was found to be 12.2 I. U. per g., while that of samples of buffalo ghee collected from villages round Coimbatore gave a value of 11.0 I. U. per g. The last group (Table VI) of miscellaneous samples of ghee probably gives a correct idea of the amount of vitamin A contained in typical samples of ghee bought in the bazaar in South India. On this basis, 2 oz. of ghee would provide about 450 I. U. of vitamin A, which is only a small fraction of requirements as these are usually estimated.

It will be seen from Tables VII to IX that there is a considerable loss of vitamin A activity when ghee or 'fortified' Margarine is used in preparations requiring prolonged heating above 100°C. Over half the vitamin A activity is lost in the case of the former, while complete destruction occurs in the case of the latter. It is, therefore, obvious that 'fortified' Margarine should not be used in frying, if its vitamin A activity is to be preserved.

SUMMARY.

1. The total vitamin A activity of ghee prepared from the milk of well-fed cross-bred cows was found to be on the average 36.5 I. U. per g.; carotene contributed approximately 31 per cent of this value.

2. Ghee prepared from milk of buffaloes fed under known conditions had an average vitamin A content of 8.8 I. U. per gramme. In general the addition of green feeds to various concentrates leads to an increase in the vitamin A content of ghee obtained from the milk of the animals concerned. The relation between the period of lactation and vitamin A content of ghee is not marked.

3. Ghee, certified to be genuine, collected from 14 centres in the Madras Presidency contained on an average 8.5 I. U. of vitamin A per g.

4. Twelve samples of blended ghee from the Ghee Heating Centre, Agra, had an average vitamin A potency of 12.2 I. U. of vitamin A per g.

5. A good proportion of the vitamin A activity of ghee, and all the vitamin A activity of 'fortified' Margarine, are lost when these are used for frying purposes.

Thanks are due to the following officers of various Governments for samples of ghee used in these investigations: (1) The Government Agricultural Chemist, Coimbatore, (2) The Government Public Health Analyst, Madras, (3) Chemist, Public Health Department, Lahore, (4) Officer-in-charge, Ghee Heating Centre, Agra, and (5) Director, Public Health Laboratory, Trivandrum.

The samples of carotene concentrates and 'fortified' Margarine were supplied by the Hindustan Vanaspati Manufacturing Co., Ltd., Bombay.

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THE DIGESTIBILITY OF CERTAIN VEGETABLE OILS AND FATS DETERMINED BY METABOLIC EXPERIMENTS ON HUMAN BEINGS.

BY

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THOUGH fats and oils in the diet are important as sources of energy and are two-and-a-quarter times as effective for this purpose as either proteins or carbohydrates, their dietetic requirement has received little attention. They are, however, recognized as essential in nutrition such as amino-acids, minerals, or vitamins owing to their content of certain indispensable unsaturated higher fatty acids (Burr, Burr and Miller, 1932).

Although the fat requirement of the human body is not definitely known nutrition experts have expressed the opinion that the minimal requirements of fat is about 50 g. and that the optimal requirement is about 120 g. per head per day (Bacharach and Drummond, 1940) of which about 60 per cent should be of animal origin. Numerous diet surveys undertaken in different parts of this country show that the average daily fat intake *per capita* is very low, barely exceeding one ounce, very little of which is of animal origin. It would appear important, therefore, that the intake of fat in India should be raised. It must be borne in mind that apart from supplying energy to the system, fats and oils have other intrinsic properties as well. 'Fat has a high satiety value, its high calorie content reduces the bulk of food consumed. It is the vehicle of fat-soluble vitamins; infants at the breast receive half of their calories in the shape of fat' (Anderson and Williams, 1937). They serve as starting materials for the production of phosphatides and cerebrosides which are essential constituents of the brain.

Lard is not popular in this country and butter-fat in amounts sufficient to supply the entire population is not available. The vegetable oils are, therefore, the only available sources of fat at present and different vegetable oils are consumed in different parts of the country. Practically no work has been done in India to determine whether there is any difference in the digestibility of the different fats and oils.

Because of the relation of fats to agriculture and their importance in the diet, a series of experiments were undertaken by the Office of Home Economics in Washington to determine the digestibility of a number of fats and oils consumed in America. Langworthy (1923) and Langworthy and Holmes (1915) studied the digestibility of a variety of common fats and oils. The results seem to indicate that the digestibility coefficient of the fats having a melting point above body-temperature (37°C.) varies inversely with the melting point. It has also been claimed that most of the common oils are from 93 to 98 per cent utilized by the human body. However, there are instances, e.g. mutton-fat (Langworthy and Holmes, 1915), oleo-stearin (Holmes, 1919), and deer-fat (Deuel and Holmes, 1922) as well as some hydrogenated fats melting between 52°C. and 60°C. (Deuel and Holmes, 1921), where values as low as 79 per cent were found for the average coefficient of digestibility. Experiments of this type with human subjects involve many difficulties, the chief of which is the fact that in general the people of any particular place are accustomed to using one type of oil or fat only, and when other types of oils are taken, they are found to cause digestive disturbances.

Hoagland and Snider (1942) have compared the digestive coefficients of lard, vegetable shortenings and mixed animal and vegetable shortenings, using rats as test animals. They found that the digestibility coefficient of lard is higher than that of the other two types

of shortenings. Studies in man by Sealock, Basinaki and Murliss (1941) determined the digestibilities of fats in mixed diets containing whole wheat and wheat bread. McCay and Paul (1938) have noted the extent of digestion of the higher melting fats in guinea-pigs. Using rats as experimental animals, the percentage of absorption of a few unheated hydrogenated and thermally-treated oils has been evaluated by Roy (1944) who has found that, excepting ground-nut oil, the absorption of all oils decreases when they are subjected to thermal treatment.

This paper records the digestibility of the various oils and fats which are used for edible purposes in different provinces of India. These include mustard, coconut, sesame and ground-nut oils and cow butter-fat, buffalo butter-fat and hydrogenated ground-nut oil (Dalda Vanaspati). Experiments with linseed oil were also tried but they proved a failure as the subjects manifested symptoms of diarrhoea on taking the oil.

EXPERIMENTAL.

The digestibility was determined by metabolic experiments on adult human subjects. The method followed in these experiments was practically the same as that followed by previous workers in this Laboratory, working on problems on metabolism (Basu and Basak, 1939). The fat in question was consumed with a basal ration of the composition : rice 600 g., pulses 100 g., vegetables 200 g.; fish (as far as possible fat-free) 70 g. The basal ration was selected as to contain as little fat as possible. It still contained, however, an appreciable quantity of fat, 8.56 g. to 10.32 g., as determined by actual analysis. The fishes used in the experiments were *icha* (*Palaemon carcinus*) and *bele* (*Glossogobius gicericis*).

Each experiment was conducted for seven days, during the first three days of which no collection of either urine or faeces was made. Faeces during the last four days were preserved with toluene and the amount of fat excreted through the faeces was determined by taking an aliquot part of the wet faeces and analysing it according to the method of Saxon (1938). Excretion of fat from the body is believed to be almost entirely in the faeces. Very little fat is excreted in the urine and so no analysis of urine for fat was made. Difficulty was often experienced in the last stages of analysis, as an emulsion of fat with petroleum ether was formed which prevented the formation of a sharp line of demarcation between the petroleum ether layer and the lower alcoholic solution. This was, however, overcome by treatment with certain electrolytes, e.g. sodium chloride.

Each fat was added in amounts of 50 g. per day. Faecal fat excretion, when the basal diet was consumed and no other oil or fat was taken, was also determined. Experimental subjects, all males, were healthy adults who were thoroughly familiar with this type of metabolic work and co-operated whole-heartedly. Experimental results are indicated in Table I. Digestibility coefficients are all corrected for fat elimination by allowing for the basal, practically fat-free, diet. The results are summarized in Table II.

TABLE I.

Showing absorption of different oils and fats by experimental subjects.

(Figures of intake and excretion represent daily averages.)

Diet.	Weight of dietary fat, g.	Weight of faecal fat, g.	Weight of absorbed fat, g.	Weight of faecal fat due to oil supplement, g.	Percentage of absorption of added fat.
SUBJECT: R. B. A., 18 years ; weight 50 kg.					
Basal	9.85	3.55	6.30
Basal and mustard oil	59.85	7.56	52.29	4.01	92
Basal and sesame oil ...	59.85	6.00	53.85	2.45	95

TABLE I—*contd.*

Diet.		Weight of dietary fat, g.	Weight of faecal fat, g.	Weight of absorbed fat, g.	Weight of faecal fat due to oil supplement, g.	Percentage of absorption of added fat.
SUBJECT: <i>R. B. A.</i> , 18 years; weight 50 kg.— <i>contd.</i>						
Basal and ground-nut oil	...	59.85	3.33	56.52	<i>Nil</i>	100
Basal and coconut oil	...	59.85	3.85	56.0	0.30	99.4
Basal and cow butter-fat	...	59.85	4.52	55.33	0.97	98
Basal and Dalda Vanaspati	...	59.85	9.31	50.54	5.76	88.5
Basal and buffalo butter-fat	...	59.85	5.07	54.08	1.52	97
SUBJECT: <i>I. B. G.</i> , 19 years; weight 52 kg.						
Basal	8.56	3.27	5.29
Basal and mustard oil	...	58.56	6.95	51.61	3.68	92.64
Basal and sesame oil	...	58.56	6.49	52.07	3.22	93.56
Basal and ground-nut oil	...	58.56	4.38	54.18	1.11	97.78
Basal and coconut oil	...	58.56	3.95	54.61	0.68	98.64
Basal and cow butter-fat	...	58.56	5.83	52.73	2.56	94.88
Basal and Dalda Vanaspati	...	58.56	8.3	50.26	5.03	89.94
Basal and buffalo butter-fat	...	58.56	6.17	52.39	2.9	94.20
SUBJECT: <i>G. C. D.</i> , 24 years; weight 60 kg.						
Basal	9.85	4.44	5.41
Basal and mustard oil	...	59.85	7.89	51.96	3.45	93.1
Basal and sesame oil	...	59.85	7.11	52.74	2.67	94.66
Basal and ground-nut oil	...	59.85	5.26	54.59	0.82	98.26
Basal and coconut oil	...	59.85	4.93	54.92	0.49	99.02
Basal and cow butter-fat	...	59.85	6.5	53.35	2.06	95.88
Basal and Dalda Vanaspati	...	59.85	9.21	50.64	4.77	90.94.
Basal and buffalo butter-fat	...	59.85	6.7	53.15	2.26	95.48

TABLE I—*concl'd.*

Diet.	Weight of dietary fat, g.	Weight of faecal fat, g.	Weight of absorbed fat, g.	Weight of faecal fat due to oil supplement, g.	Percentage of absorption of added fat.
SUBJECT: H. P. D., 26 years; weight 48 kg.					
Basal	10.32	3.59	6.73
Basal and mustard oil	60.32	4.2	56.12	0.61	98.78
Basal and sesame oil ...	60.32	2.74	57.58	Nil	100
Basal and ground-nut oil	60.32	2.88	57.44	Nil	100
Basal and coconut oil	60.32	3.23	57.09	Nil	100
Basal and cow butter-fat	60.32	3.67	56.65	0.08	99.84
Basal and buffalo butter-fat	60.32	3.86	56.46	0.27	99.46

TABLE II.

Showing the mean digestibility of different fats.

(Average of experiments on four subjects.)

Type of fat.	Percentage digestibility.
Mustard oil	94.13
Sesame oil	95.8
Ground-nut oil	99.0
Coconut oil	99.28
Cow butter-fat	97.15
Buffalo butter-fat	97.53
Dalda Vanaspati	89.8

RESULTS AND DISCUSSION.

Consideration of the results indicates that in all cases but one the coefficient of digestibility exceeds 94 per cent and there is no great difference in the digestibilities of the animal and vegetable oils and fats which are liquid at ordinary room temperature. Results with 'Vanaspati', however, indicate a slightly lower value. This is to be expected because hydrogenation raises the melting point of the fatty acids which in turn lowers the absorption coefficients of fats and oils. An upper limit (preferably below 40°C.) for the melting point of edible hydrogenated fats and oils might prove desirable. These results have also been arrived at by Langworthy and Holmes (1915).

It seems reasonable to conclude that practically all natural fats and oils are absorbed to the fullest extent, and for energy production they may probably be considered as almost equally efficient. Some oils are less tolerated by certain people than others. Before we can say whether or not an oil or fat is suitable for use in human dietary we must know not only its percentage digestibility but also other properties, as for example, tolerance, toxic effects, etc. Only further work can tell whether they possess identical physiological properties. Their effects on stimulating growth and on calcium and phosphorus metabolism should also be considered. The results of investigations on these lines will be shortly communicated.

SUMMARY.

The digestibility of certain vegetable oils used for edible purposes in different parts of India, viz. mustard, coconut, sesame and ground-nut oils, of a sample of hydrogenated ground-nut oil (Dalda Vanaspati) and also of cow and buffalo butter-fats (ghee) has been determined by metabolic experiments on four human experimental subjects. It was concluded that the natural fats and oils are almost completely utilized by the human body (94 to 99 per cent), while hydrogenation appears to lower the digestibility to some extent.

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THE RATE OF ABSORPTION OF DIFFERENT FATS AND OILS.

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IN evaluating the importance of natural oils and fats for edible purposes, two points of fundamental importance are their digestibility coefficients and rates of absorption; the first indicates the extent to which they are absorbed in the system and the second determines the amount of a particular fat or oil that is absorbed in a definite time. The latter point should be determined specially because a fat or oil which is absorbed to a maximum amount in the least time should always be preferred. The first problem has been dealt with by some investigators (Langworthy, 1923; Langworthy and Holmes, 1915; Deuel and Holmes, 1921) and with few exceptions, fats having a low melting point have been found to be almost completely absorbed from the gastro-intestinal tract of normal men. This has also been confirmed by the present authors (Basu and Nath, 1946), the fats and oils used in the experiments being those which are commonly used in different parts of India for edible purposes.

On the contrary, very few studies have been carried out on the rate of absorption of the different fats and oils especially of those which are commonly used in India. Steenbock, Irwin and Weber (1936) employing a technique described by Irwin, Steenbock and Templin (1936), which is merely a modification of the technique used by Cori (1925) for determining the rate of absorption of sugars, found the extent of absorption of a number of fats over a 6-hour period. Deuel, Hallman and Leonard (1940) determined the rates of absorption of another batch of fats and oils differing in chemical composition, which are commonly used in America.

The present paper deals with the study of the comparative rates of absorption of several fats and oils that are used in different parts of India, namely, mustard, coconut, sesame and ground-nut oils, and cow butter-fat.

EXPERIMENTAL.

The technique was practically the same as that of previous workers mentioned above, with some modifications at various points. The fats were fed by stomach-tubes to rats, weighing from 225 g. to 250 g. and fasted previously for 48 hours. During this period of fasting, water was given *ad libitum*, but the rats lost 8 to 10 per cent of their body-weight. The stomach-tube was inserted by holding the rat by the neck and placing a forceps between the teeth. In earlier experiments light anaesthesia was employed. A glass receptacle was fitted to the end of the stomach-tube. The melted fat was poured into it from a pipette and was then introduced into the stomach by means of a gentle push with a hand-pump fitted at the end of the glass receptacle.

The small amount of oil remaining on the receptacle and stomach-tube was recovered in each case and duly allowed for in calculating the exact quantity introduced into the stomach.

Amounts of fat absorbed after periods of 2, 4 and 6 hours were determined. At the end of the definite interval the rats were killed, the intact gastro-intestinal tract was removed and the fats remaining unabsorbed were determined by water and petroleum-ether extraction according to the procedure of Irwin, Steenbock and Templin (*loc. cit.*).

A set of preliminary experiments was also performed to determine the extent of recovery of fat immediately after it has been introduced by the stomach-tube. The results are

indicated in Table I. It has been found that the mean percentage of recovery lies between 96 and 99 per cent and often it is 100 per cent. No correction is, therefore, necessary for any amount of fat which might escape extraction from the gastro-intestinal tract.

It has been found by Irwin, Steenbock and Templin (*loc. cit.*) that sex plays no important rôle in the rate of absorption and nearly identical results were obtained by using either male or female rats. For this reason rats of both sexes have been used. It has also been found by the same workers that rats of different sizes exhibit different rates of absorption with the same oil and for the same time interval, i.e. the rate of absorption depends on the surface area of the stomach, intestine, etc., and so in all the experiments rats were chosen which were nearly of the same weight. The amount of fat remaining in the gastro-intestinal tract after a fast of 48 hours was also found to be insignificant.

Tables II to VII give the rate of absorption of the various oils and fats. Table VIII summarizes the rates of absorption of all fats investigated. Table IX shows the effect of the amount of fat administered on the rate of absorption of coconut oil for a period of 2 hours.

TABLE I.
Percentage of recovery of fat immediately after administration.

Weight of coconut oil administered, g.	Amount in the tubes, etc., g.	Total amount administered, g.	Amount recovered, g.	Percentage of recovery.	Mean percentage of recovery.
0.7868	0.0953	0.6915	0.6645	96.1	97.46
	0.0753	0.7115	0.6987	98.2	
	0.0819	0.7049	0.6873	97.5	
	0.1059	0.6809	0.6734	98.9	
	0.0983	0.6885	0.6651	96.6	

TABLE II.
Rate of absorption of mustard oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount remaining in the tube, g.	Exact amount administered, g.	Amount recovered, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1.6836	0.1534	1.5302	1.1264	0.4038	26.4	27.6±0.871
		0.1253	1.5583	1.1593	0.3990	25.6	
		0.1628	1.5208	1.0539	0.4669	30.7	
4	1.6836	0.1593	1.5243	0.6280	0.8963	58.8	56.3±0.752
		0.1432	1.5404	0.7070	0.8334	54.1	
		0.1523	1.5313	0.6738	0.8575	56	
6	1.6836	0.1234	1.5602	0.5570	1.0032	64.3	64 ±0.867
		0.1038	1.5798	0.6130	0.9668	61.2	
		0.1534	1.5302	0.5126	1.0176	66.5	

TABLE III.
Rate of absorption of coconut oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount remaining in the tube, g.	Exact amount administered, g.	Amount recovered, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1.5754	0.0987	1.4767	0.9997	0.4770	32.3	36 \pm 1.063
		0.1076	1.4678	0.9262	0.5416	36.9	
		0.1036	1.4718	0.9007	0.5711	38.8	
4	1.5754	0.0943	1.4811	0.8156	0.6655	44.8	42.7 \pm 0.740
		0.1237	1.4517	0.8681	0.5836	40.2	
		0.1053	1.4701	0.8365	0.6336	43.1	
6	1.5754	0.0873	1.4881	0.6920	0.7961	53.5	49.8 \pm 1.171
		0.0763	1.4991	0.7690	0.7301	48.7	
		0.0857	1.4897	0.8000	0.6897	46.3	

TABLE IV.
Rate of absorption of olive oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount recovered from stomach-tube, etc., g.	Exact amount administered, g.	Amount extracted from stomach and intestine, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1.6152	0.0856	1.5296	1.0285	0.5011	39.3	41.1 \pm 0.478
		0.0793	1.5359	0.8724	0.6635	43.2	
		0.0823	1.5329	0.9075	0.6254	40.8	
4	1.6152	0.0921	1.5231	0.8164	0.7067	46.4	48.9 \pm 0.719
		0.0758	1.5394	0.7605	0.7789	50.6	
		0.0843	1.5309	0.7700	0.7609	49.7	
6	1.6152	0.1053	1.5099	0.5874	0.9225	61.1	59.8 \pm 0.857
		0.1042	1.5110	0.5802	0.9308	61.6	
		0.0970	1.5182	0.6566	0.8616	56.7	

TABLE V.
Rate of absorption of ground-nut oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount recovered from stomach-tube, etc., g.	Exact amount administered, g.	Amount extracted from stomach and intestine, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1.6992	0.1123	1.5869	1.0236	0.5633	35.5	35.13 ± 0.534
		0.0913	1.6079	1.0725	0.5354	33.3	
		0.1023	1.5969	1.0124	0.5845	36.6	
4	1.6992	0.0856	1.6136	0.7987	0.8149	50.5	52 ± 0.428
		0.0972	1.6020	0.7626	0.8394	52.4	
		0.1123	1.5869	0.7443	0.8426	53.1	
6	1.6992	0.0957	1.6035	0.6398	0.9637	60.1	59.9 ± 0.480
		0.0823	1.6169	0.6257	0.9912	61.3	
		0.0834	1.6158	0.6636	0.9522	58.3	

TABLE VI.
Rate of absorption of sesame oil.

Time (hours).	Weight of oil taken (2 c.c.), g.	Amount recovered from stomach-tube, etc., g.	Exact amount administered, g.	Amount extracted from stomach and intestine, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1.7039	0.0750	1.6289	1.0412	0.5877	36.1	36.2 ± 0.335
		0.0829	1.6210	1.0164	0.6046	37.3	
		0.0836	1.6203	1.0500	0.5703	35.2	
4	1.7039	0.0953	1.6086	0.8815	0.7271	45.2	46.1 ± 0.273
		0.0875	1.6164	0.8583	0.7581	46.9	
		0.0913	1.6126	0.8340	0.7786	46.3	
6	1.7039	0.0859	1.6180	0.6909	0.9271	57.3	57.3 ± 0.191
		0.1031	1.6008	0.6931	0.9077	56.7	
		0.0989	1.6050	0.6757	0.9293	57.9	

TABLE VII.

Rate of absorption of cow butter-fat.

Time (hours).	Amount administered (2 c.c.), g.	Amount recovered from gastro-intestinal tract, g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
2	1.3084	$\left\{ \begin{array}{l} 0.7988 \\ 0.7538 \\ 0.7654 \end{array} \right.$	$\left\{ \begin{array}{l} 0.5096 \\ 0.5546 \\ 0.5430 \end{array} \right.$	$\left\{ \begin{array}{l} 39.0 \\ 42.0 \\ 41.5 \end{array} \right.$	40.8 ± 0.510
4	1.3084	$\left\{ \begin{array}{l} 0.7362 \\ 0.6288 \\ 0.6742 \end{array} \right.$	$\left\{ \begin{array}{l} 0.5722 \\ 0.6796 \\ 0.6342 \end{array} \right.$	$\left\{ \begin{array}{l} 43.4 \\ 52.1 \\ 48.4 \end{array} \right.$	47.97 ± 1.390
6	1.3084	$\left\{ \begin{array}{l} 0.5306 \\ 0.5098 \\ 0.5688 \end{array} \right.$	$\left\{ \begin{array}{l} 0.7778 \\ 0.7986 \\ 0.7396 \end{array} \right.$	$\left\{ \begin{array}{l} 59.4 \\ 61.3 \\ 56.7 \end{array} \right.$	59.13 ± 0.735

TABLE VIII.

Percentage of different oils absorbed after 2, 4 and 6 hours.

Type of oils.		PERCENTAGE OF DIFFERENT OILS ABSORBED AFTER		
		2 hours.	4 hours.	6 hours.
Mustard oil	...	27.6 ± 0.871	56.3 ± 0.752	64.0 ± 0.867
Coconut oil	...	36.0 ± 1.063	42.7 ± 0.740	49.8 ± 1.171
Olive oil	...	41.1 ± 0.478	48.9 ± 0.719	59.8 ± 0.858
Ground-nut oil	...	35.13 ± 0.534	52.0 ± 0.428	59.9 ± 0.480
Sesame oil	...	36.2 ± 0.335	46.1 ± 0.273	57.3 ± 0.191
Cow butter-fat	...	40.8 ± 0.511	47.97 ± 1.390	59.13 ± 0.735

TABLE IX.

Effect of amount of oil administered on the rate of absorption.

(Oil taken—coconut.)

Amount administered, g.	Amount recovered from stomach-tube, g.	Exact amount administered, g.	Amount extracted from stomach and intestine, etc., g.	Amount absorbed, g.	Percentage of absorption.	Mean percentage of absorption.
0.7877	0.0856	0.7021	0.4739	0.2282	32.5	32.3 ± 0.355
	0.1050	0.6827	0.4704	0.2123	31.1	
	0.0987	0.6890	0.4593	0.2297	33.3	
1.5754	0.0987	1.4767	0.9997	0.4770	32.3	36 ± 0.106
	0.1076	1.4678	0.9262	0.5416	36.9	
	0.1036	1.4718	0.9007	0.5711	38.8	
2.3631	0.0973	2.2658	1.3480	0.9178	38.3	38.8 ± 0.159
	0.0759	2.2872	1.3823	0.9049	39.3	
	0.1021	2.2610	1.3737	0.8873	38.8	

ANALYSIS OF THE DATA.

The probable error of the mean results is indicated in the tables. Analysis of the mean values obtained 2 hours after administration reveals that the difference of 13.2 ± 1.01 between the absorption of cow butter-fat and mustard oil is clearly significant. The difference of 8.4 ± 1.371 between the absorption of coconut and mustard oils as also the difference of 4.8 ± 1.175 between cow butter-fat and coconut oil are significant.

Four hours after administration the difference of 4.3 ± 0.86 between the absorption of mustard and ground-nut oils is significant. The difference of 9.2 ± 1.27 between coconut and ground-nut oils 6 hours after administration is significant being 7.24 times its probable error.

RESULTS AND DISCUSSION.

From the results it is clear that there are appreciable differences between the rates of absorption of the various fats and oils, especially when we consider the results of absorption in the first few hours. It is seen that 2 hours after administration, olive oil and cow butter-fat are the most rapidly absorbed, while mustard oil is the least absorbed. This is to be expected since the former varieties contain a larger percentage of short-chain fatty acids and as these are somewhat water-soluble, they are likely to be absorbed more rapidly than the long-chain fatty acids. The results, however, become striking 4 hours after the fat is administered when it is seen that mustard oil is absorbed to the greatest extent. The difference between the rates of absorption of olive, sesame and ground-nut oils and cow butter-fat is not marked at this time and at the 6-hour period they are practically identical except in the case of coconut oil which shows less absorption than the others. The results of our previous investigation (Basu and Nath, *loc. cit.*) have shown that, during the 24-hour period, the absorption of all the natural fats and oils is almost complete and it is probably from the results at the 6-hour period that the percentage of absorption of all the fats and oils except coconut oil tends to become equal.

In contrast to the findings of Deuel, Hallman and Leonard (*loc. cit.*) and also of Steenbock, Irwin and Weber (*loc. cit.*) it has been found that the rate of absorption of oil (coconut oil in our case) increases slightly with an increase in the amount of oil administered (see Table IX). With increased amounts of the oil fed the absolute amount absorbed, however, increased in all their experiments. The comparisons of the above-mentioned authors were made 3 hours after the administration of the oil, i.e. when the absorption was well on its way, while in our experiments comparisons were made 2 hours after administration. The effect of concentration may possibly change after this interval.

SUMMARY.

The rates of absorption of mustard, coconut, olive, ground-nut and sesame oils and of butter-fat have been compared using rats (225 g. to 250 g. weight) as test animals. The fats were fed by the stomach-tube and at the end of definite intervals (2, 4 or 6 hours) the rats were killed, the intact gastro-intestinal tract was removed and the fats remaining unabsorbed were determined.

Two hours after administration olive oil and butter-fat were found to be the most rapidly absorbed fats. After 4 hours mustard oil is absorbed to the greatest extent and the others show almost equal rates of absorption. After 6 hours the fats are all almost equally absorbed, the absorption of coconut oil being slightly less than that of the others.

With an increase in the amount of coconut oil administered the rate of absorption of the oil was found to be slightly increased.

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THE EFFECT OF DIFFERENT FATS ON CALCIUM UTILIZATION IN HUMAN BEINGS.

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THE problem of the influence of dietary fat on calcium utilization has received considerable attention with somewhat conflicting results. Nearly all investigators have, however, worked with rats and have shown that the addition of fat to a fat-free diet has often a favourable influence on calcium utilization. Variations in experimental conditions and dietary régime have, however, led to diverse conclusions. Knudson and Floody (1940) and Jones (1940) furnished clear-cut evidence that moderate levels of fat in the diet favoured the utilization of calcium. Earlier investigators in this field, viz. Cronheim and Müller (1908), Bahrtdt (1910), Rothberg (1907) and Birk (1907), found that as the amount of fat in the diet increased above a moderate level, calcium soaps in the stool also increased. Bosworth, Bowditch and Giblin (1918, 1918a) concluded that the presence of soluble ionized calcium in the intestine determined the extent of soap formation, while loss in the faeces depended not only upon the amount of calcium soaps formed but also upon their nature, calcium oleate being much more soluble in the normal intestinal fluids than was calcium palmitate or calcium stearate. It was shown by Givens (1917) that in the case of low fatty acid utilization, e.g. with ethyl palmitate and palmitic acid, the loss of calcium was increased.

Some recent publications also point to the same results. Jones (1942) and French (1942) have independently proved that in rats dietary fat increases the acidity of the intestinal contents, in particular in the jejunum and lower ileum, and that this change in acidity is responsible for increased absorption of calcium and phosphorus. Booth, Henry and Kon (1942) have found that on a high calcium, low phosphorus diet calcification of bones is definitely aided by the presence of fat in the diet. The effect is not evident with a low calcium, high phosphorus diet.

Telfer (1923, 1930) has outlined the mechanism as follows: Free fatty acids dissolve calcium phosphate with the formation of calcium soaps which are then absorbed. He has also pointed out that if fats and phosphates are both low, calcium carbonate may be excreted in an alkaline stool. The form in which calcium is eliminated from the intestinal tract is, therefore, associated with the pH of the intestinal contents as a consequence of the effect of acidity upon solubility.

It was, however, indicated by the work of Boyd, Crum and Lyman (1932) that all the calcium soaps are not absorbed equally. The three soaps investigated by them were calcium oleate, calcium palmitate and calcium stearate. The first showed the highest percentage of absorption and the last-named the least.

When fats and oils are taken, they are mostly resolved, by the action of fat-splitting enzymes, into a mixture of fatty acids in the intestine. Each fat has a definite composition peculiar to itself so far as its fatty acid make-up is concerned. As already pointed out some of these fatty acids then form salts with calcium. The work of Boyd (*loc. cit.*) has definitely shown that the percentage of utilization of different calcium soaps is not equal. This being the case, it may be possible that a difference in the nature of the calcium soaps formed out of the fatty acids of different fats and oils may result in a difference in the amount of absorption of calcium.

The purpose of the present investigation was to find out whether there was any relation between the nature of the oil fed and the total amount of calcium absorbed. An attempt was also made to find whether any relation existed between the nature of the oil fed and phosphorus metabolism.

EXPERIMENTAL.

The experimental procedure was the same as that reported in previous communications by Basu and his co-workers (Basu and Basak, 1939; Basu, Basak and De, 1941). An experimental diet of the composition rice 600 g., pulses 70 g., fish 60 g. and vegetables 200 g. together with 60 g. of the oils was consumed by healthy male subjects for 7 days. The first 3 days were observed as a preliminary period and the urine and faeces of each subsequent period of 4 days were collected and analysed and the mean of daily analytical figure is represented. The analytical methods for the estimation of calcium and phosphorus were the same as employed in the previous investigations of Basu *et al.* (1939, 1941).

Tables I to VI show the metabolism data of calcium and phosphorus with different oils as dietary fat with different subjects:—

TABLE I.

Calcium metabolism with different fats.

SUBJECT: G. C. D., 24 years, weight 60 kg.

Diet.		Food Ca (mg.).	Urinary Ca (mg.).	Faecal Ca (mg.).	Total Ca excretion (mg.).	Balance (mg.).
Basal and fat-free	253	52.6	193.7	246.3	6.7
Basal and mustard oil	...	262	43.1	123.5	163.6	98.4
Basal and coconut oil	...	247.6	47.2	208.3	255.5	—7.9
Basal and ground-nut oil	...	259.7	35.6	182.1	217.7	42
Basal and sesame oil	...	243.2	32.4	182.7	215.1	28.1
Basal and cow butter-fat	...	241.7	36.9	176.6	212.5	39.2

TABLE II.

Calcium metabolism with different fats.

SUBJECT: A. C. D., 18 years, weight 50 kg.

Diet.		Food Ca (mg.).	Urinary Ca (mg.).	Faecal Ca (mg.).	Total Ca excretion (mg.).	Balance (mg.).
Basal and fat-free	205.5	38.7	180.6	219.3	—13.8
Basal and mustard oil	...	209.5	31.5	132	163.5	46
Basal and coconut oil	...	217.2	78.1	250.8	328.9	—111.7
Basal and ground-nut oil	...	206	33.7	169.8	203.5	2.5
Basal and sesame oil	...	221	26.5	161.2	187.7	33.3
Basal and cow butter-fat	...	213.6	34	182.7	216.7	—3.1

TABLE III.

Calcium metabolism with different fats.

SUBJECT: I. B. G., 19 years, weight 53 kg.

Diet.	Food Ca (mg.).	Urinary Ca (mg.).	Faecal Ca (mg.).	Total Ca excretion (mg.).	Balance (mg.).
Basal and fat-free ...	177	42.9	133.9	176.8	0.2
Basal and mustard oil	187	34.7	65.2	99.9	87.1
Basal and coconut oil	180.8	75	228	303	-122.2
Basal and sesame oil	180.8	36.6	115.7	152.3	28.5
Basal and ground-nut oil	186	25.3	149.6	174.9	11.1
Basal and cow butter-fat	193.6	31.3	123.7	155	38.6

TABLE IV.

Calcium metabolism with different fats.

SUBJECT: S. C. D., 19 years, weight 52 kg.

Diet.	Dietary Ca* (mg.).	Urinary Ca (mg.).	Faecal Ca (mg.).	Total Ca excretion (mg.).	Balance (mg.).
Basal and fat-free ...	485	165.8	351.3	517.1	-32.1
Basal and mustard oil	487.3	62.5	330.6	393.1	94.2
Basal and coconut oil	482.7	88.4	508.2	596.6	-113.9
Basal and ground-nut oil	503	79	312	391	112
Basal and sesame oil	492.5	127.9	328.6	456.5	36
Basal and cow butter-fat	512	45.3	189	234.3	277.7
Basal and buffalo butter-fat ...	497	53.4	261.3	314.7	182.3

* The comparatively larger intake of calcium in this experiment is due to inclusion of leaves of *Cucurbita pepo* (pumpkin) in the vegetables taken. These leaves contain 240 mg. to 300 mg. of calcium per 100 g. of the moist leaf. A paper on the availability of calcium in *Cucurbita pepo* leaves is being communicated for publication.

TABLE V.

Phosphorus metabolism with different fats.

SUBJECT : I. B. G., 19 years, weight 53 kg.

Diet.		Dietary P (mg.).	Urinary P (mg.).	Fæcal P (mg.).	Total P excretion (mg.).	Balance (mg.).
Basal and fat-free	387.5	195.5	145	340.5	47
Basal and mustard oil	...	375.6	154.2	139.3	293.5	82.1
Basal and coconut oil	...	392.8	163.7	161.2	324.9	67.9
Basal and sesame oil	...	385.7	162.5	146.1	308.6	77.1
Basal and ground-nut oil	...	373.5	150.2	151.6	301.8	71.7
Basal and cow butter-fat	...	395.7	171.2	152.3	323.5	72.2

TABLE VI.

Phosphorus metabolism with different fats.

SUBJECT : S. C. D., 19 years, weight 52 kg.

Diet.		Dietary P (mg.).	Urinary P (mg.).	Fæcal P (mg.).	Total P excretion (mg.).	Balance (mg.).
Basal and fat-free	721	276	215	491	230
Basal and mustard oil	...	746	189	174	363	383
Basal and coconut oil	...	715.1	221	189	410	305.1
Basal and ground-nut oil	...	732	207	232	439	293
Basal and sesame oil	...	726.3	239	235	454	272.3
Basal and cow butter-oil	...	718	186	162	348.2	369.8
Basal and buffalo butter-fat	...	705.5	193.8	227	420.8	284.7

DISCUSSION.

The data prove definitely two things: (1) firstly, the presence of fat in the diet greatly favours the absorption of calcium and phosphorus in the body. Addition of fat in the diet lowers the excretion of the calcium and phosphorus by the urinary tract to a great extent. The excretion of phosphorus in the faeces is also decreased to some extent and the elimination of calcium through the faeces is reduced to a much greater extent in all cases except with coconut oil. Probably the fatty acids form absorbable calcium soaps with calcium which would otherwise have been excreted as insoluble calcium carbonate, (2) all fats are not equally efficient in bringing about favourable calcium absorption and indeed on basal diet and coconut oil there occurs a drainage of calcium from the body. The intake of coconut oil as the main dietary fat cannot be recommended in view of the results obtained in this investigation. When coconut oil was used as the sole dietary fat, the experimental subjects were invariably in negative calcium balance. The faecal excretion of calcium was greatly increased and in three cases out of four exceeded the intake of calcium. With this oil in the diet the urinary elimination of calcium was generally higher than with other oils and butter-fat. The reason for this peculiar behaviour of coconut oil is not clear. Probably some particular calcium soaps that are being produced in the intestine as a result of digestion of the coconut oil are not absorbed at all. Further work is in progress in order to elucidate this point.

SUMMARY.

The influence of the dietary fat on the calcium and phosphorus metabolism in four human subjects has been investigated. Inclusion of mustard, sesame or ground-nut oil or of butter-fat in the diet greatly favoured the absorption of calcium and phosphorus and the utilization of the minerals was better than in the absence of added fat. With coconut oil, however, the elimination of calcium through the faeces was greatly increased, the faecal calcium being in three cases greater than the amount of dietary calcium. The urinary excretion of calcium on coconut oil diet was also generally higher than with other fats. The use of coconut oil as the main dietary fat cannot be recommended in view of these findings.

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COMPARATIVE VALUE OF BUTTER-FATS AND VEGETABLE OILS FOR GROWTH.

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It was realized long ago that diets containing appreciable amounts of fat are superior to those comprised almost entirely of carbohydrate and protein (Burr and Burr, 1930). But the question whether all fats and oils are equally efficient still remains unanswered. Sure (1941) found that diets containing 15 per cent of lard, butter-fat, crisco, olive oil or wheat-germ oil were equally effective in promoting lactation and for the general well-being of the animals. Hoagland and Snider (1942) compared diets containing 5 or 15 per cent of various lards or manufactured shortenings. The average growth rates of rats for all of the lards were essentially the same as the average for all of the shortenings. No known physical or chemical properties of the individual fat were found to be correlated with its dietary value.

Schantz, Elvehjem and Hart (1940) studied the effect of different fats of vegetable origin on growth, general health and reproductive capacity by rat-feeding experiments and compared their results with that obtained with butter-fat. For short-time experiments they found butter-fat to be superior to others in every respect.

Such comparison between butter and other fats has been made from time to time in different laboratories with conflicting results. Boer (1941) postulated a new growth factor in butter-acids because his rats grew more rapidly on butter than on olive oil. Contrary to this is the finding of Euler, Euler and Salberg (1942) that Margarine promotes better growth than butter-fat. Boutwell, Geyer and Hart (1943) on the other hand found appreciably less growth on corn oil than on butter-fat homogenized into skim milk. So far it appears that no clear answer as to the cause of the difference between different types of fats has been obtained.

Again, opinions vary as to the optimum level of fat in the diet at which it produces maximum growth. Maynard and Rasmussen (1942) showed with mixed diets of natural foods that the one containing the higher percentage of fat gave superior growth of young ones. Contrary opinions have also been expressed by many who are of the opinion that fat above a certain level produces deleterious effects by throwing an unnecessary strain on such organs as the liver.

The present paper deals with the comparative nutritive values of different fats and oils, e.g. mustard, coconut, sesame and ground-nut oils and cow and buffalo butter-fats, so far as their effect on the growth promotion of young albino rats is concerned. A comparison has also been made among three levels of fat intake in the diet, viz. 3, 6 and 9 per cent.

EXPERIMENTAL.

The general plan followed in the investigation was to feed rats on diets all the constituents of which were the same excepting the fat portion which was different for different groups. The diet was similar in composition to those which are generally used in nutritional experiments containing all the essential food ingredients in optimum as well as balanced amounts. It consisted (in parts per 100) of ether-extracted egg-protein 10, salt mixture (Osborne and Mendel, 1913) 4, agar 1, sucrose 9, calcium carbonate 1, fat or oil 6 and

starch 69. Vitamins A and D were supplied in the form of Adexolin and vitamin B complex as yeast extract. All diets were made up at weekly intervals and were stored in the refrigerator.

Using a diet of this composition as the basis, six different diets in each of which the fat fraction was different as already mentioned, were fed to respective groups of rats during an experimental period of eight weeks. Young albino rats one month old and weighing from 40 g. to 50 g. were used in these investigations. Four animals were taken in each group, care being taken in the group distribution to minimize both litter and sex variations. Each rat was kept in an individual cage and a weighed amount of well-cooked diet was supplied together with water *ad libitum*. The weight of the diet supplied to each rat was accurately recorded and due correction was applied for any amount of diet that was not consumed by the animals. Bi-weekly records of the weights of individual rats were kept. The first week was observed as a preliminary period to eliminate the effects of diets taken by the rats previously and the weight of the rats and the amount of diet consumed by them and used in the calculation shown in the tables were recorded from the second week.

Table I shows the comparative rates of growth of rats on the different oils and fats already named, growth being measured as increase in weight of rats per week per g. of fat ingested.

ANALYSIS OF THE DATA.

The probable error of the mean growths has been indicated in the Tables. Analysis of these mean values shows that the difference of 0.25 ± 0.0263 between the growth with mustard and ground-nut oils is distinctly significant, the difference being 9.5 times the probable error. The difference between mustard and coconut oils, 0.05 ± 0.0217 , is only 2.30 times the probable error and is, therefore, not significant. The difference between butter-fat and vegetable oils is obviously significant.

In the experiments on the effect of concentration of butter-fat on growth the difference of 0.49 ± 0.0529 between the 3 and 6 per cent levels is significant. In the case of 6 and 9 per cent levels the difference 0.02 ± 0.037 is not significant, the probable error being greater than the difference.

RESULTS AND DISCUSSION.

From the results it is clear that, of all the oils and fats studied, butter-fat induced the maximum degree of growth and sesame oil the least. The reason for such differences in the growth-stimulating properties of different oils and fats is little understood. Of course it is true that food consumption was slightly increased in the cases of rats on butter-fat in comparison with those on vegetable oils. Better growth with these may be due to the general effects of increased amounts of food materials but in the case of the rats on diets containing vegetable oils, where food consumption was nearly equal in all cases, an appreciably better growth was observed with ground-nut oil than that with other oils.

The presence in fats and oils of some particular fatty acids which can produce more growth than others, might be one possible explanation of this different behaviour of fats and oils. It is very doubtful whether this explanation can stand scrutiny. It has been stated by Burr and Burr (*loc. cit.*), as well as by a number of other investigators, that the daily requirement of fatty acids essential in nutrition, namely, linoleic and linolenic acids, are exceedingly small. According to the analysis recorded by Jamieson (1932), linoleic acid is present in a number of vegetable oils and fats. In ground-nut oils it constitutes nearly 25 per cent and in sesame oil about 35 per cent of the total fatty acids. Other natural oils and fats also contain linoleic acid in greater or less amounts.

On the contrary, investigations have shown that it is the saturated fatty acid portion of fats and oils which induced better growth in comparison with the unsaturated fatty-acid portion. Thus the content of saturated fatty acids might serve as the criterion for evaluating the growth-inducing value of different fats and oils. Chemical analysis of the oils and fats, however, shows no marked difference in their content of saturated fatty acids and so some other factor must be sought to account for the difference in growth-promoting value of different oils and fats.

EXPERIMENTAL RESULTS.

TABLE I.

Comparative rates of growth of young rats on diets composed of different oils and fats at 6 per cent level of fat intake.

Type of fat or oil.	Initial weight of rats, g.	Final weight, g.	Total increase in weight, g. (for 7 weeks).	Increase in weight per week, g.	Total food intake, g.	Food intake per week, g.	Fat intake per week, g.	Increase in weight per week per g. of fat intake.	Mean increase in weight per week per g. of fat intake.
Mustard ...	45	94.5	49.5	7.07	504	72	4.32	1.64	1.54 ± 0.0196
	43	90	47	6.7	518	74	4.44	1.51	
	48	98	50	7.14	561	80.1	4.81	1.49	
	50	104.5	54.5	7.79	595	85	5.10	1.52	
Coconut ...	42	92	50	7.14	575	82.1	4.93	1.45	1.49 ± 0.009
	40	89.5	49.5	7.07	548	78.3	4.7	1.5	
	54	107	53	7.6	597.3	85.3	5.12	1.48	
	48	102.5	54	7.73	589.1	84.17	5.05	1.53	
Sesame ...	47.5	89.5	42	6.00	549.5	78.5	4.71	1.28	1.32 ± 0.00
	53	98.5	45.5	6.5	565.6	80.8	4.85	1.34	
	50.5	98.5	48	6.86	589.1	84.17	5.05	1.32	
	48	91.5	43.5	6.21	560	80	4.8	1.32	
Ground-nut ...	51.5	120	68.5	9.8	619.5	88.5	5.31	1.85	1.79 ± 0.0176
	46.5	115	68.5	9.8	610	87.1	5.27	1.83	
	49	109.5	60.5	8.64	607.6	86.8	5.21	1.72	
	51	111.5	60.5	8.64	593.6	84.8	5.09	1.76	
Buffalo butter-fat ...	55	127	72	10.3	614.6	87.8	5.27	1.95	1.98 ± 0.0148
	49	119.5	70.5	10.07	620	88.6	5.32	1.90	
	47	120	73	10.4	607.8	86.8	5.21	2.0	
	52	125	73	10.4	609.8	87.1	5.26	1.99	
Cow butter-fat ...	47	130	83	11.86	623.7	89.1	5.35	2.21	2.25 ± 0.0202
	43	124	81	11.5	618.8	88.4	5.31	2.18	
	48	132	84	12.0	616	88	5.28	2.29	
	51.5	139.5	88	12.6	630.5	90.1	5.41	2.33	

TABLE II.

Growth of young rats on various levels of butter-fat intake.

Diet.	Initial weight of rats, g.	Final weight, g.	Total increase in weight, g. (for 8 weeks).	Increase in weight per week, g.	Total food intake, g.	Food intake per week, g.	Fat intake per week, g.	Increase in weight per week per g. of fat intake.	Mean increase in weight per week per g. of fat intake.
Experiment abandoned after 5 weeks as the rats consumed diets in insufficient amounts.									
Fat-free ...	51	87	62.5	7.81	649.6	81.2	4.87	1.6	1.72 ± 0.0406
	43	78		9.94	670.5	83.8	5.03	1.9	
	50	77.5		8	654.3	81.8	4.91	1.63	
	48	79		8.75	660	82.5	4.95	1.77	
3 per cent	49	111.5	96	12	681.5	85.2	5.12	2.35	2.21 ± 0.0284
	47	126.5	91	11.37	686.4	85.8	5.14	2.21	
	53.5	117.5	86	10.75	664.1	83	4.98	2.16	
	50.5	120.5	85.5	10.7	670.5	83.8	5.02	2.13	
6 per cent	43	139	90	11.25	684	85.5	5.13	2.2	2.19 ± 0.0246
	51.5	142.5	85	10.62	676.1	84.5	5.07	2.09	
	49.5	135.5	88.5	11.06	656.2	82	4.92	2.25	
	47	132.5	89	11.12	667.9	83.5	5.01	2.22	
9 per cent	49.5	139.5	90	11.25	684	85.5	5.13	2.2	2.19 ± 0.0246
	42.5	127.5	85	10.62	676.1	84.5	5.07	2.09	
	51	139.5	88.5	11.06	656.2	82	4.92	2.25	
	47.5	136.5	89	11.12	667.9	83.5	5.01	2.22	

Attempts have also been made to explain this difference with respect to the content of unsaponifiable material but no decisive conclusion was reached. The trend of modern researches suggests that fats and oils contain some so far unidentified factor which is responsible for inducing different growth rates.

Table II shows that of the three levels of fat intake studied, viz. 3, 6 and 9 per cent, maximum growth is induced at the 6 per cent level whereas a slightly lower value, which is not statistically significant, is obtained in the case of the 9 per cent level. A rather poor growth is obtained when the fat is given at 3 per cent level in the diet. Further experiments are required to decide whether administration of fat at a level higher than 9 per cent would have an adverse effect on growth.

SUMMARY.

Butter-fat fed at 3, 6 and 9 per cent levels to young rats induced maximum growth at the 6 per cent level; the growth was poor at the 3 per cent level. The difference in growth at the 6 and 9 per cent levels is not significant.

With mustard, coconut, sesame or ground-nut oil or with cow or buffalo butter-fats fed to young rats, all at 6 per cent level, maximum growth—an increase in weight of about 12 g. per week and 2.25 g. per week per g. of fat intake—was obtained with cow butter-fat. The growth with buffalo butter-fat was slightly less. The vegetable oils gave less growth. Of the vegetable oils ground-nut oil gave the greatest (1.79 g. per week per g. of fat intake) and the sesame oil (1.32 g. per week per g. of fat intake) the least growth. Mustard and coconut oils gave a growth intermediate between ground-nut and sesame oils and the difference between mustard and coconut oils was not significant.

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DESTRUCTION OF VITAMIN B₁ OF SOME VEGETABLES DURING COOKING AND THE EFFECT OF COOKING ON FREE AND COMBINED VITAMIN B₁ OF SOME FOODSTUFFS.

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THE importance of aneurin or thiamin in biological reactions in the prevention and cure of beri-beri and various other diseases has proved it to be an essential factor in the diet. The thiamin content of various foodstuffs has been determined by many workers in India and abroad. Such a knowledge is essential in evaluating the thiamin content of a particular diet and in suggesting diets adequate with regard to that vitamin. Thiamin is, however, heat labile, and cooked foods are generally taken by human beings. Thus, a mere knowledge of the thiamin content of raw foodstuffs is not enough in assessing the actual intake of thiamin. The study of the effect of cooking on the retention of thiamin is, therefore, a subject of great importance.

Arnold and Elvehjem (1939) studied the effect of processing on the thiamin content of beef-lung, beef-kidney and beef-spleen. The influence of processing on the thiamin content of rice was shown by Kik and van Landingham (1943). The effect of canning on the thiamin content of some vegetables was studied by Harris, Procter, Goldblith and Brody (1940), Grzhivo and Kondrashova (1940), Farrel and Fellers (1942) and by Clifcorn and Heberlein (1944).

The effect of cooking on the retention of thiamin in fresh and frozen peas was shown by Barnes, Tressler and Fenton (1943), in peas by Rose and Phipard (1937) and by Johnston, Schauer, Rapaport and Denel (1943). Retention of thiamin in meat after cooking and roasting was studied by McIntire, Schweigert, Henderson and Elvehjem (1943), Schweigert, McIntire and Elvehjem (1943) and by Cover, McLaren and Pearson (1944).

The thiamin content of five varieties of potatoes after cooking was estimated by Wertz and Weir (1944). Aughey and Daniel (1940) studied the effect of cooking on potatoes and spinach. Hoff (1933) reported that there was a loss of more than 50 per cent of the vitamin B₁ in spinach cooked by ordinary household methods. Nagel and Harris (1943) estimated the loss of thiamin in restaurant cooking and holding of some vegetables. Vitamin losses in large-scale cooking in the case of peas, beans, spinach, carrot, potatoes and cabbage were studied by Heller, McCay and Lyon (1943). A comprehensive study of the effect of cooking on the retention of thiamin in common Indian vegetables is lacking and hence the necessity of undertaking such an investigation seems to be a subject of importance.

Much thought has also been given to the mode of destruction of thiamin during cooking. Sherman and Burton (1926) observed that tomato juice lost 20 per cent of its thiamin content through heating at 100°C. for four hours at the natural pH of 4.28 but that when the pH was raised to 9.2 the destruction was 60 to 70 per cent in one hour. Factors involved in the destruction of vitamin B₁ by heat are little understood since losses of vitamin B₁ may occur in one foodstuff but not in another under similar process of cooking. In attempts to elucidate this point we felt that it would be interesting to study the effect of cooking on the 'free B₁' and 'bound B₁', i.e. cocarboxylase contents of foodstuffs.

EXPERIMENTAL.

The fluorometric method based on Jansen's (1936) thiochrome reaction as developed by Wang and Harris (1941, 1942) was used for the estimation of thiamin. The method suggested by the Committee on Vitamin Fortification of the American Association of Cereal Chemists as modified by Moyer and Tressler (1942) was followed for the extraction and liberation of the vitamin from the vegetables. As recommended by Brown, Hamm and Harrison (1943), takadiastase was used instead of clarase for the enzymic liberation of the vitamin and incubation was effected at 52°C. for two hours. In the case of flesh foods digestion was effected with papain as well as with takadiastase. As only a very limited supply of iso-butyl alcohol was available, normal butyl alcohol was tested and found to be satisfactory. Farrer (1941) also showed that the normal alcohol could be used for the estimation of thiamin. Recovery of added thiamin usually ranged from 75 to 100 per cent.

The vegetables and other foodstuffs were all collected from local markets. Only edible portions were taken for analysis. There was no preliminary washing. The thiamin content was determined in the raw as well as in the cooked portion of the same sample. Cooking was done by boiling for half an hour in distilled water. The whole of the boiled extract together with the boiled portion was used in the estimation. The effect of cooking on total, free and combined B₁ content of foodstuffs is indicated in Tables I and II :—

TABLE I.
Thiamin content of vegetables before and after cooking.

Common name (English and Bengali).	Botanical name.	Thiamin content before cooking, in µg. per 100 g.	Thiamin content after cooking, in µg. per 100 g.	Percentage of vitamin B ₁ retained after cooking.
Amaranth (tender) ...	<i>Amaranthus gangeticus</i>	24.0	18.0	75.0
Dudhkôchu (black-tuber) ...	<i>Xanthosoma violaceum</i>	33.0	29.0	87.0
Borbôti ...	<i>Vigna catjang</i>	60.0	50.0	83.0
Bitter gourd (small) ...	<i>Momordica charantia</i>	42.0	30.0	71.0
Bottle-gourd (Lâu) ...	<i>Lagenaria vulgaris</i>	10.0	5.0	50.0
Bottle-gourd leaves and tendril	20.0	17.5	87.5
Brinjal ...	<i>Solanum melongena</i>	30.0	9.0	30.0
Carrot ...	<i>Daucus carota</i>	29.0	22.0	76.0
Cabbage ...	<i>Brassica oleracea</i> var. <i>capitata</i> .	50.0	30.0	60.0
Châl kumra ...	<i>Benincasa cerifera</i>	12.5	5.0	40.0
Drumstick (or Indian horse-radish) ...	<i>Moringa oleifera</i>	2.1	1.8	86.0
Dhenki sâk ...	<i>Diplazium esculentum</i>	6.0	4.0	66.6
Garlic ...	<i>Allium sativum</i>	33.0	24.0	74.0
Jhinga ...	<i>Luffa acutangula</i>	30.0	22.5	75.0
Jack, tender pumpkins ...	<i>Artocarpus integrifolia</i>	20.0	8.0	40.0
Jute leaves ...	<i>Corchorus capsularis</i>	21.0	12.0	57.0
Kumra (pumpkin), sweet ...	<i>Cucurbita</i> sp.	42.0	19.8	47.0
Kumra (pumpkin) leaves	60.0	39.0	65.0
Motor shunti (peas) ...	<i>Pisum sativum</i>	28.0	18.0	64.0
Onion ...	<i>Allium cepa</i>	44.0	32.0	72.7
Papaya (raw) ...	<i>Carica papaya</i>	18.1	11.0	60.7
Potato ...	<i>Solanum tuberosum</i>	50.4	38.0	75.4
Plantain flower ...	<i>Musaparakdisiaca</i>	20.0	Nil	Nil
Plantain stem	3.8	2.5	66.0
Plantain, green	64.3	10.0	16.0
Pepper, green ...	<i>Capsicum frutescens</i>	12.5	9.0	72.0
Patôl ...	<i>Trichosanthes dioica</i>	48.0	22.4	46.7
Puin sâk ...	<i>Basella rubra</i>	30.0	12.5	41.3
Potato (sweet) ...	<i>Ipomoea batatas</i>	40.0	25.0	62.5
Seem ...	<i>Dolichos lablab</i>	28.0	18.0	64.3
Spinach (sweet) ...	<i>Beta vulgaris</i>	15.0	7.5	50.0
Tomato, green ...	<i>Lycopersicum esculentum</i>	25.0	18.5	74.0
Turnip (coloured) ...	<i>Brassica rapa</i>	30.0	18.0	59.4
Thalkuri ...	<i>Hydrocotyle asiatica</i>	16.5	7.5	45.4
Heléncha ...	<i>Enhydra fluctuans</i>	5.0	Nil	Nil

TABLE II.

Showing the effect of cooking on the free B_1 and coenzyme content of foodstuffs
(in $\mu\text{g. per } 100 \text{ g.}$).

Common name.	Botanical name.	FREE B_1 .			TOTAL B_1 .			COENZYME:	
		Raw.	Boiled.	Per cent destroyed on cooking.	Raw.	Boiled.	Per cent destroyed on cooking.	Raw.	Boiled.
Onion <i>Allium cepa</i>	12.8	1.2	90.6	44.0	32.0	27.2	31.2	30.4
Patol <i>Trichosanthes dioica</i>	30.0	5.2	82.6	48.0	22.4	53.3	18.0	17.2
Mung dhal (raw)	... <i>Phaseolus mungo</i>	240.0	80.0	66.6	450.0	300.0	33.3	210.0	220.0
Bengal gram (without husk)	... <i>Cicer arietinum</i>	150.0	48.0	68.0	450.0	345.0	23.3	300.0	297.0
Black gram	... <i>Phaseolus radiatus</i>	91.0	28.0	69.2	210.0	141.0	31.4	119.0	116.0
Soya bean (Dacca)	... <i>Glycine hispida</i>	96.0	Nil	100.0	330.0	240.0	27.2	234.0	240.0
Egg (duck's)	128.0	53.0	50.7	132.0	53.0	57.6	4.0	0.0
Liver (ho-goat)	38.4	12.0	68.7	76.8	52.8	32.2	38.4	40.8
Mout (" breast muscle)	14.4	Nil	100.0	55.2	43.2	21.7	40.8	43.2

RESULTS AND DISCUSSION.

Table I shows that all the vegetables investigated lost some of their thiamin on cooking. This loss seems to be due to the destruction of the vitamin as there was no other preliminary treatment. Lunde, Kringstad and Olsen (1940) stated that in cooking the danger of loss of vitamin was by extraction rather than by heat-destruction. Other investigators, a reference to whose work has also already been made, are of opinion that there is little or no destruction of B₁ by heat—the loss being entirely due to preliminary extraction or blanching or leaching. The present investigation shows that the loss in cooking seems to be entirely due to heat-destruction as there was no other preliminary treatment in the case of the foodstuffs. The entire amount of vitamin B₁ will of course become available only if the water in which the vegetable is cooked is not thrown away.

From the results in Table II it is apparent that the loss of free vitamin B₁ ranges from 50 to 100 per cent, while the 'bound B₁' or the cocarboxylase content of the foodstuff is practically constant before and after cooking. As all the foodstuffs were cooked at their natural pH, the variation in the percentage destruction of the free B₁ may be explained from the observations of Beadle, Greenwood and Kraybill (1943) who reported that stability of thiamin to heat is a function not only of pH but also of the electrolyte system involved. McIntire and Frost (1944) have studied the effect of concentration of amino-acids and related compounds on the stability of thiamin. Sure and Ford (1943) studied the effect of incubation at 37.5°C. on the stability of thiamin and riboflavin in cows' milk and observed that a quarter of the destructive potency could be attributed to the minerals in the milk. Thus, the variation in the percentage destruction of free B₁ in Table II may quite possibly be due not only to variations in the pH within the tissues but also to variations in the electrolyte systems and possibly to other factors such as the protein systems involved. The results presented in Table II clearly point to the fact that the bound or the cocarboxylase portion of the vitamin present in the foodstuffs is not affected by heat and in the case of the nine representative foodstuffs reported in Table II the cocarboxylase content remains practically constant after boiling the foodstuffs for half an hour.

SUMMARY.

The thiamin retention of thirty-five vegetables after cooking has been determined by the thiochrome method.

A portion of the thiamin content of all the vegetables investigated is destroyed by cooking. The average retention of thiamin after cooking of the vegetables is about 60 per cent.

The percentage destruction of free B₁ and of total B₁ in the case of some foodstuffs has also been studied. It is found that only a portion (50 to 100 per cent) of the free B₁ is destroyed by heat, while the 'bound B₁' or the cocarboxylase portion of the foodstuffs remains practically unaffected.

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TABLE I.

*Average weekly relative growth rates (per cent) of 18 rats,
9 males and 9 females, fed on a poor rice diet
with supplements of butter and ragi.*

Proportion of ragi in the rice diet.							
Butter.	Nil.		1/6		1/3		TOTAL.
	F	M	F	M	F	M	
Nil ...	3.18	6.78	8.01	10.57	11.63	13.30	53.47.
0.4 g.	3.48	2.65	7.26	6.98	7.97	8.29	36.63
0.8 g.	0.12	1.79	7.32	3.88	8.88	0.95*	22.94
TOTAL	18.00		44.02		51.02		113.04

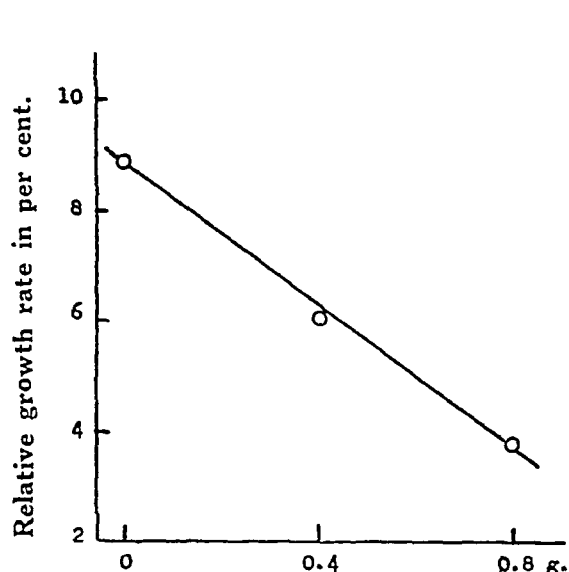
* This rat suffered from a wound in the leg.

Means per group.

Proportion of ragi in the rice diet.					
Butter.	Nil.		1/6		GRAND MEANS.
	F	M	F	M	
Nil ...	5.0	6.78	8.01	10.57	8.9
0.4 g.	3.1	2.65	7.26	6.98	6.1
0.8 g.	0.9	1.79	7.32	3.88	3.8
GRAND MEANS	3.0		7.3		6.3

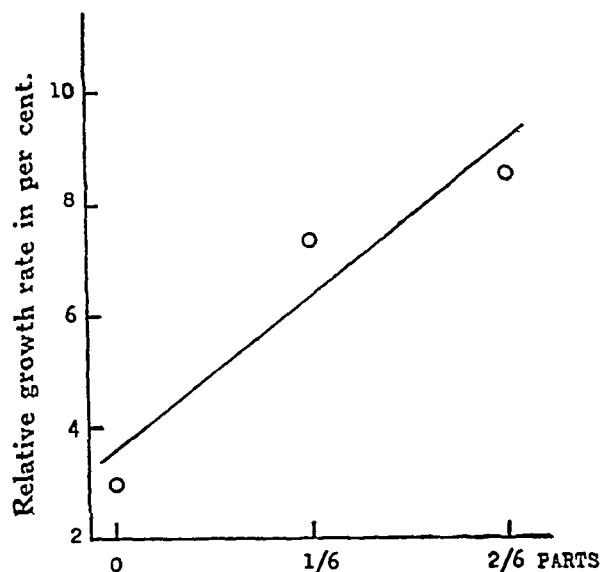
The results show clearly that butter exerts a definitely inhibiting effect on growth, the effect being more marked the greater the amount given, and that ragi definitely augments

growth. These effects are shown in Graphs 1 and 2, as regressions of relative growth rates, the regression on butter being clearly linear, that on ragi predominantly linear.



Daily supplement of butter.

GRAPH 1.



Ragi content in rice diet.

GRAPH 2.

Influence of butter and ragi on growth of rats fed on a basal rice diet. The curves show the regression of the average weekly growth rate on the amount supplied of butter and ragi respectively; each point based on 6 rats (cf. Table II).

The analysis of variance (Table II) shows that both the negative effect of butter and the positive effect of ragi are highly significant, and that there is no significant interaction between them, i.e. the presence of ragi did not facilitate the utilization of additional fat nor prevent its inhibiting effect on growth.

TABLE II.
Analysis of variance of findings in Table I.

Items.	S. Sq.	D. F.	M. Sq.	V. R.	Probability.
<i>Butter :—</i>					
Linear regression ...	78.03	1	78.03	12.33	0.01 — 0.001
Quadratic regression ...	0.25	1	0.25	0.04	0.90 — 0.80
<i>Ragi :—</i>					
Linear regression ...	90.75	1	90.75	14.34	0.01 — 0.001
Quadratic regression ...	10.24	1	10.24	1.62	0.30 — 0.20
<i>Ragi and butter interactions</i> ...	9.31	4	2.33	0.37	0.20 — 0.05
Sex ...	0.41	1	0.41	0.076	0.80 — 0.70
Error ...	50.62	8	6.33
TOTAL ...	239.61	17

S. Sq. = Sum of squares; D. F. = Degrees of freedom; M. Sq. = Mean square or variance; V. R. = Variance ratio.

DISCUSSION.

It is probable (as judged by unpublished experiments with different South Indian cereals as partial substitutes or supplements to the poor rice diet) that the most important factor in ragi in augmenting growth in rats on the poor rice diet is its high calcium content. Calcium lactate has been shown to augment the growth rate markedly but not to counteract the adverse effect of additional butter in the diet (Mason *et al.*, *loc. cit.*). The present experiment shows that neither the proteins nor other factors present in ragi are adequate to give that 'protection' which is provided by technical casein for the utilization of fat with the poor rice diet.

SUMMARY.

1. Butter and ragi (*Eleusine coracana*) were given as supplements in increasing amounts separately as well as simultaneously to young rats on a basal poor rice diet.

2. Butter had a marked inhibiting effect on growth directly proportional to the amount given.

3. Ragi substituted for part of the rice had a marked stimulating effect on growth directly proportional to the amount given.

4. Butter and ragi when given simultaneously exerted their effects independently of each other, i.e. ragi did not counteract the negative effect of butter.

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THE ABSORPTION OF FATS FROM THE HUMAN INTESTINE.

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INTRODUCTION.

THE rate of absorption of fat may be measured either directly by determining at intervals the amount of fat remaining in the alimentary tract after feeding fat or indirectly by following the appearance of fat in the blood stream. Steenbock, Irwin and Weber (1936) delivered by a tube 1.5 c.c. melted fat into the stomach of anæsthetized rats. The animals were killed at fixed intervals and the fat remaining in the gastro-intestinal tract estimated by suitable methods. They observed that in anæsthetized rats (1) partially hydrogenated vegetable oils were absorbed as rapidly as lard or corn oil and (2) that butter oil, halibut-liver oil and cod-liver oil were absorbed uniformly at a more rapid rate than lard, corn oil or partially hydrogenated fats. Deuel, Hallman and Leonard (1940) found no consistent differences in the rates of absorption of hydrogenated cotton-seed oil, 'wintered' cotton-seed oil, butter-fat or coconut-oil.

Irwin, Steenbock and Templin (1936) used the chylomicron technique of Gage and Fish (1924) for studying the rate of absorption of fats. Fasted rats were fed 0.5 c.c. fat by stomach tube. A small drop of blood from the end of the rat's tail was touched with a glass-slide and examined under dark-ground illumination. The authors observed, however, that there were many errors in the technique and comparable results could not be obtained.

Rapid changes in the fat content of the blood due to the absorption of fat are not easily demonstrable by methods involving chemical analysis as relatively large amounts of blood are required for this purpose. Recently, the use of chylomicrographs which show the number of fat particles in the blood with a standard dark-ground microscopic technique has made serial investigations of the blood-fat easier. The changes demonstrated by this method are sufficiently great to be significant but the quantitative relationship can at present be regarded only as relative (Frazer, 1940).

Frazer and Stewart (1937, 1939) determined the most suitable technique for obtaining particle counts. They also carried out simultaneous estimations of neutral fat, cholesterol and ultramicroscopic particles in human blood after a fatty meal. They found that the neutral fat curve ran parallel to that of the particle count, whereas the blood cholesterol curve remained up and was still rising an hour or more after the other curves had returned to the basic level. Thus, the blood-fat curve rose and fell with that of the particle count and the timing of the basic levels and peaks of these two curves coincided exactly. The particles only increased after a fatty meal; carbohydrate and proteins did not affect the particle count. Elkes, Frazer and Stewart (1939) later showed that the composition of particles seen in normal human blood under dark-ground illumination was fatty and that there might be a layer of absorbed globulin at the oil-water interface.

Frazer and Stewart (1937) observed that the lipæmia following a meal containing butter-fat occurred within 1½ hours of ingestion and reached a maximum in 2 to 3 hours, the curve

returning to the resting level in $4\frac{1}{2}$ hours. The reliability of the chylomicron count in the determination of blood lipoids and in studying the rates of fat absorption has been confirmed by Cöoper and Lusk (1942).

Frazer's (*loc. cit.*) method thus appeared to be suitable for the study of the comparative rates of absorption of different edible fats. The investigation could be carried out on human beings under normal physiological conditions. The quantity of blood required would be small and the results quickly obtained.

EXPERIMENTAL.

Subjects and the standard meal.

The subjects were all normal and healthy members of the staff and were between 20 and 40 years of age. They had their last meal before 9-30 p.m. on the previous evening and came to the laboratory early in the morning with an empty stomach. Blood samples were taken before the standard meal and then at hourly intervals for the next 5 to 7 hours.

The standard meal was generally 1 oz. of the fat to be investigated along with 2 oz. of bread or *chapattis* (unleavened bread) made from 2 oz. of wheat flour. In addition a cup of tea with a little milk in it might or might not be taken. The meal was eaten within a period of 5 minutes.

Collection of blood and examination of specimen.

(a) *Specimen of blood.*—The specimen was collected in a fine capillary tube after finger puncture. The capillary tube was then sealed at one end and the blood was allowed to clot in the tube which was incubated at 37°C . for 2 hours. After one hour, the clot was gently separated at its upper end and left to contract.

(b) *Preparation of the specimen.*—A tiny drop of the separated serum was transferred by a narrow pipette to a clean slide of the correct thickness so as to give the thinnest possible film under the coverslip.

The thickness of the slides was 1.0 mm. which was checked by passing the slides through a special gauge; and the coverslips were gauge 0 with a diameter of 15 mm.

(c) *Optical system.*—A $1/12$ inch oil immersion apochromatic objective, $\times 20$ eyepiece, cardioid dark-ground condenser with a pointolite lamp as illuminant were used. A suitable stop was placed in the eyepiece limiting the field of observation.

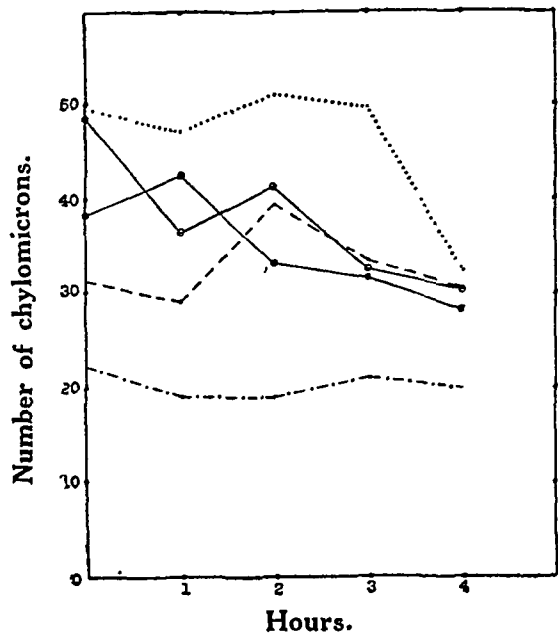
(d) *Counting.*—In the early part of the investigation two persons counted 10 fields each. Usually the difference between the averages of the two observers did not exceed 5 to 10 per cent. Later on, however, the counts made by one of the authors were occasionally checked for their accuracy.

In examining a specimen, there are three definite planes, as described by Frazer (*loc. cit.*), that can be focused. Two consist of still particles which are adsorbed to the coverslip or slide and are not numerous in a good preparation. Between these two there is seen a layer of moving particles and it is this plane which is examined. The particles are of two types, dull and bright. No alteration of lighting or focus changes a dull particle into a bright particle or vice versa.

Blank experiments.

In order to test whether eating bread alone contributed to the rise in the particle count, blank experiments were carried out. The subjects were given 2 oz. bread only and blood samples were examined before and after its ingestion.

Graph 1 represents the chylomicrograph of the above experiments. In this and all the subsequent graphs the subjects have been indicated by identical signs which are given in the legend below Graph 1 :—



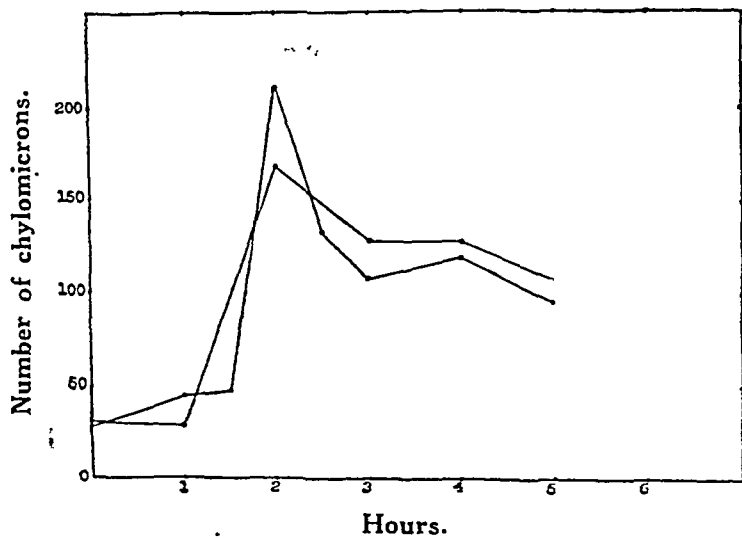
GRAPH 1.—Chylomicrograph after ingestion of 2 oz. bread only. Five subjects.

Legend :—

N. G. N.	• ——— •	V. N. P.	○ ——— ○
P. K. D.	- - - - -	N. M. C.
K. S. R.	- . - . -		

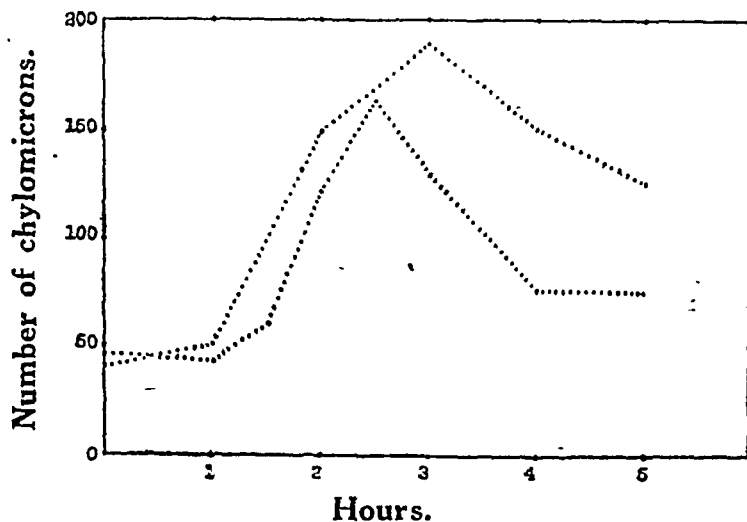
These subjects are indicated by similar signs in all the subsequent figures.

Most of the experiments were carried out in duplicate at intervals of a week or more. The results obtained with any one subject were reproducible within certain limits, e.g. the



GRAPH 2.—Chylomicrograph after ingestion of 1 oz. ghee in test meal. Two observations on subject N. G. N.

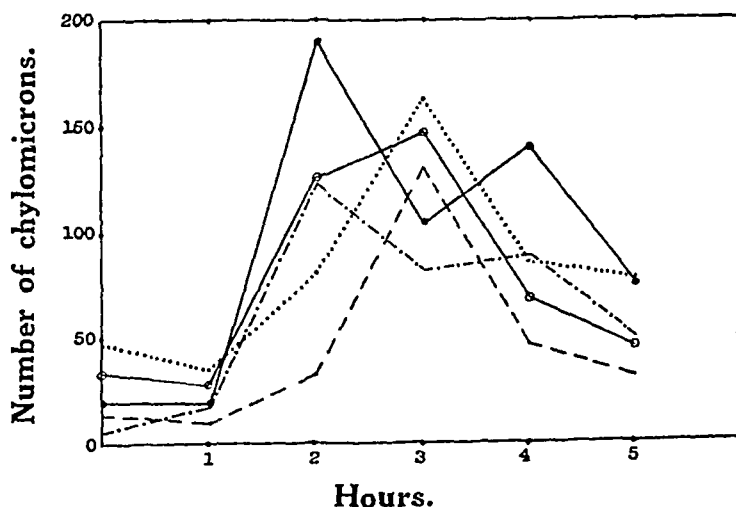
feeding of the same quantity of a particular fat in the standard meal to the same subject gave a maximum chylomicron count which although not identical was attained at the same interval after feeding as had been observed earlier. The results of two such experiments are illustrated in Graphs 2 and 3 :—



GRAPH 3.—Chylomicrograph after ingestion of 1 oz. ghee in test meal. Two observations. Subject N. M. C.

Experiments with edible fats.

Butter.—Experiments with 30 g. of fresh dairy butter with 2 oz. bread were carried out on five volunteers. The results are shown in Graph 4. The maximum count was reached in between 2 and 3 hours. In most of the experiments the particle count returned to the low level by the 5th hour. No rise in the particle count has been observed in the first hour except with the subject K. S. R.



GRAPH 4.—Chylomicrograph after ingestion of 1 oz. butter in test meal. Five subjects.

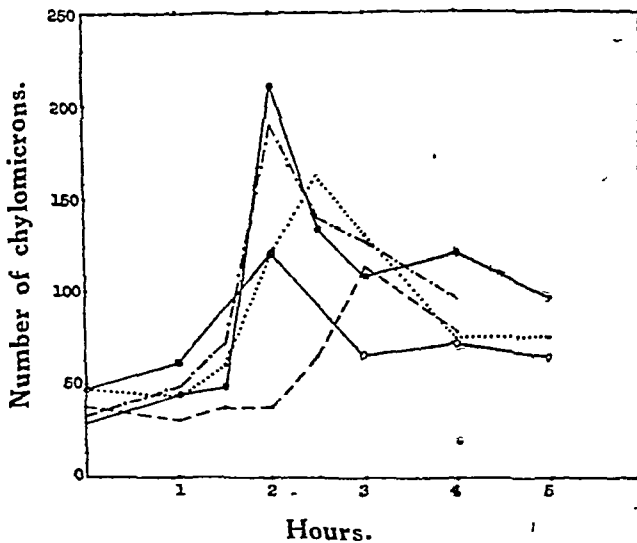
Ghee.—In India, unlike in the Western countries, ghee prepared from butter is used for the purposes of eating as well as in cooking. In the following experiments, ghee was prepared from fresh dairy butter by gently boiling off the water. The standard meal consisted of 1 oz. ghee and 2 oz. bread. In some experiments *Chapattis* prepared from 2 oz. of wheat flour were eaten instead of 2 oz. bread along with the ghee. In Table I are given the results of these experiments from which it will be clear that either bread or *chapatti* could be eaten with the fat without altering the trend of absorption. Graph 5 illustrates the absorption of ghee from the intestines.

TABLE I.

Chylomicron counts after the ingestion of 1 oz. ghee with 2 oz. bread or chapattis made from 2 oz. of wheat flour.

Hours.	N. G. N.		P. K. D.		N. M. C.	
	Bread.	Chapatti.**	Bread.	Chapatti.**	Bread.	Chapatti.**
0	30.5	28.6	29.7	37.3	40.6	46.3
1	27.1	44.0	32.6	29.9	50.5	43.4
1½	...	47.3	...	37.5	...	60.2
2	168.0	210.0	98.7	37.1	149.0	121.0
2½	...	133.0	...	65.0	...	163.0
3	128.5	108.5	126.0	112.8	189.0	130.5
4	128.0	121.0	114.3	79.0	151.5	76.0
5	107.8	96.0	114.0	...	124.8	75.0

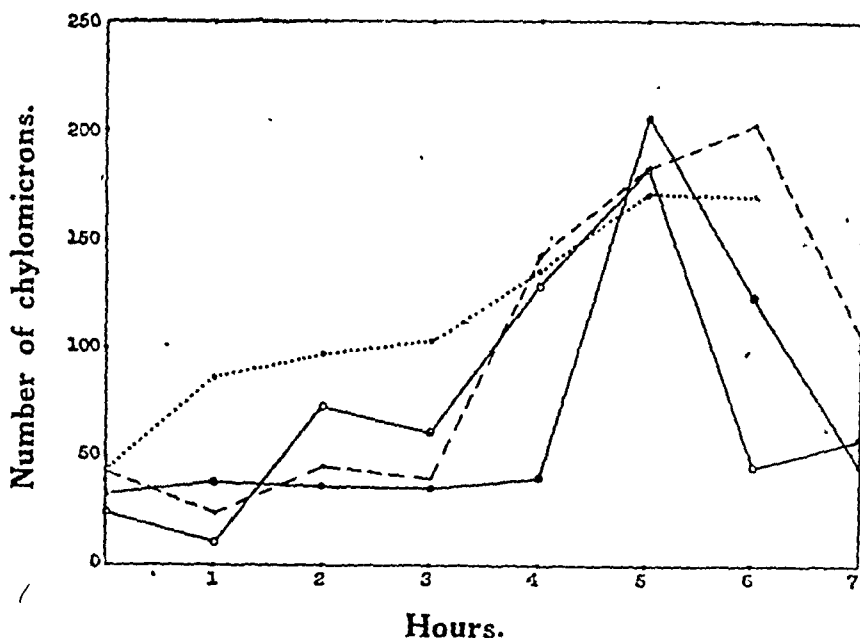
Note.—**The results given in columns (Table 1) marked with double asterisk at the top have been incorporated in Graph 5. These are in addition to those of the other two volunteers not included in this table.



GRAPH 5.—Chylomicrograph after ingestion of 1 oz. ghee in test meal. Five subjects.

Ground-nut (Arachis hypogaea) oil.—Cold oil, as such, is seldom eaten and it was found difficult to take 1 oz. of the ground-nut oil with 2 oz. bread. All the subjects, however, tolerated this standard meal although it gave rise to a certain amount of temporary nausea in some. One of the subjects actually vomited after the ingestion of the standard

meal and the experiment had to be discarded. Graph 6 illustrates the results of these experiments :—

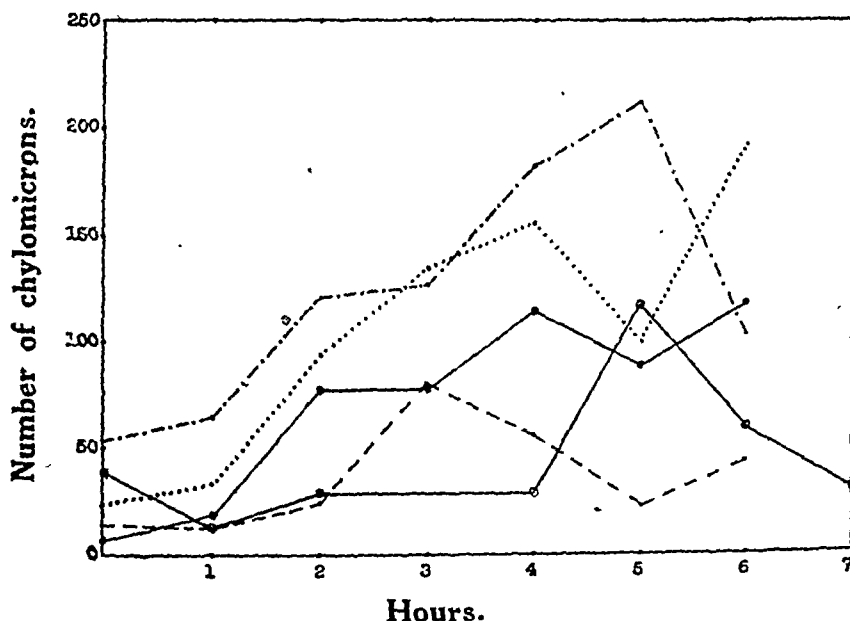


GRAPH 6.—Chylomicrograph after ingestion of 1 oz. ground-nut oil in test meal. Four subjects.

The maximum count of the particles is obtained by about the 5th hour in all cases except the subject P. K. D. where it is seen to be in the 6th hour. After the 6th hour, with subjects N. G. N. and V. N. P., the count has reached almost the basal level but in subject P. K. D. it is still lagging behind.

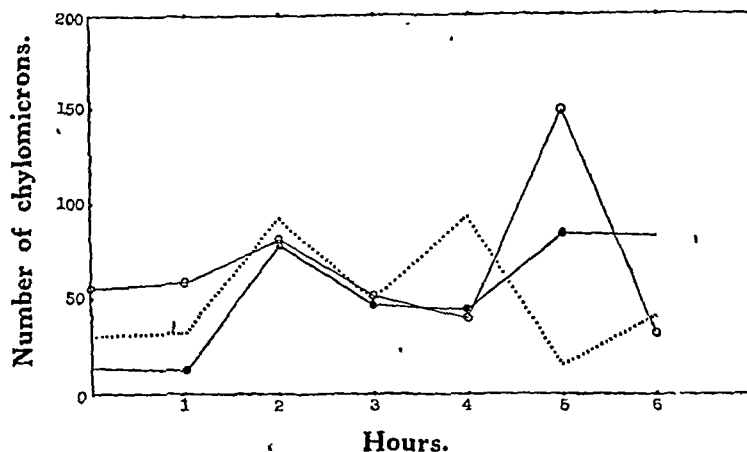
Hydrogenated ground-nut oil.—In order to find out whether hydrogenation of an oil influenced its rate of absorption, a sample of a well-known brand of refined and partially hydrogenated ground-nut oil obtained from the local market was used. The standard meal consisted of 1 oz. of this product with 2 oz. of bread.

In one subject the maximum count was obtained in 3 hours and in two in 5 hours. In the remaining two subjects a high level of chylomicron count was reached in the 4th hour. This was, however, followed by a fall and again by a rise in the 6th hour.



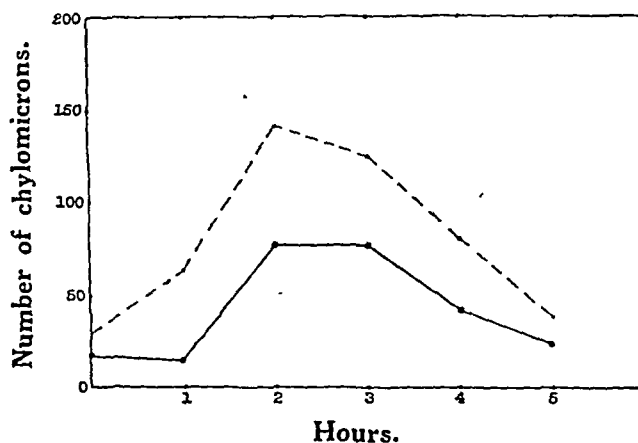
GRAPH 7.—Chylomicrograph after ingestion of 1 oz. Vanaspati in test meal. Five subjects.

Sesame (Sesamum indicum) oil.—Sesame oil is used in several parts of India for cooking purposes. The standard meal in the experiment with the sesame oil consisted of 1 oz. oil along with 2 oz. bread. An increase in the particle count was noticed after 2 hours followed by a diminution in the next hour. There was a second maximum reached after 4 hours (see Graph 8). A similar phenomenon was observed with the subject N. G. N. and N. M. C. in the case of hydrogenated ground-nut oil.



GRAPH 8.—Chylomicrograph after ingestion of 1 oz. sesame oil in test meal. Three subjects.

Coconut (Cocos nucifera) oil.—Unfortunately, only two experiments could be carried out with this oil as no more volunteers could be induced to eat the standard meal containing this oil. In Graph 9 are plotted the results of these two experiments :—



GRAPH 9.—Chylomicrograph after ingestion of 1 oz. coconut oil in test meal. Two subjects.

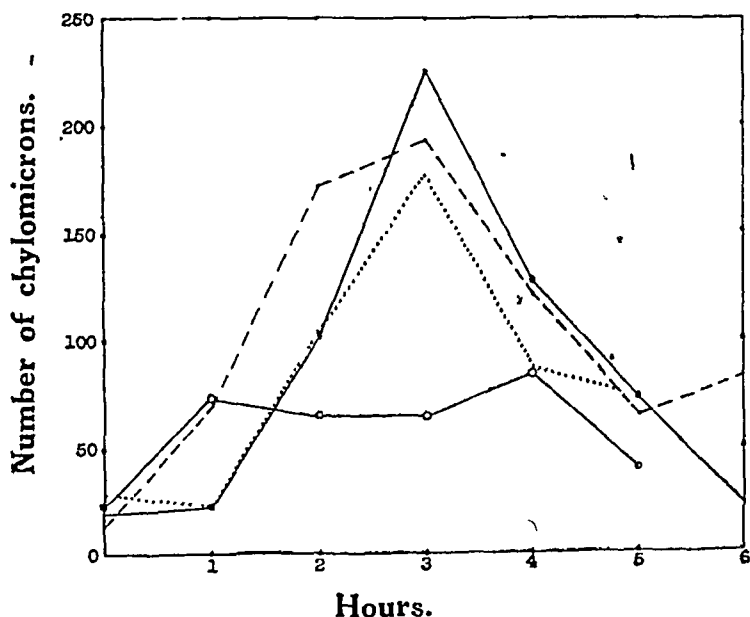
In both the experiments the maximum particle count was reached within a short period of 2 hours in contrast with that of the other vegetable oils.

Frying in ghee.—Apart from the quantity of fat eaten as such, the amount used for cooking purposes in the daily dietaries is not inconsiderable. The edible substance, when fried in fat, remains in an undecomposed condition for a longer time. A small coating of fat is formed all over the substance. This coating slows down the action of enzymes in the stomach and it takes a longer time for fried substances to leave it. Whether this delayed emptying of the stomach contents affects the absorption of fat which has been used for frying is not known. Heat does cause some transformation in the constitution of foodstuffs. Ray (1944) has studied the thermal effects on fat and has found that no change occurs in

the chemical constitution of fat though a change in the viscosity takes place. In order to determine the rate of absorption of fat after frying the following experiments were carried out :—

Two ounces of wheat flour were well mixed with water and a little ghee (taken from a weighed portion of 1 oz.) and kneaded into a dough. The dough was then rolled out into 6 or 7 thin circular slices which were fried in ghee (1 oz. less that taken for preparing the dough). It took about 10 minutes for frying these seven *purees*, one by one. Almost all the ghee was incorporated in these *purees* which constituted the standard meal.

The results of these experiments are represented in Graph 10. The maximum count was reached by about 3 hours in three subjects and in one by the 4th hour. It appears, therefore, that heating the fat to 240°C. to 250°C. for 10 minutes has very slightly delayed its rate of absorption.



GRAPH 10.—Chylomicrograph after ingestion of *purees* prepared from 2 oz. wheat flour fried in 1 oz. ghee at 240°C. to 250°C.

Experiments with a smaller amount of fat in the test meal.

So far experiments had been carried out with 1 oz. of fat but the ingestion of this quantity was often inconvenient owing to resultant nausea and a possibility of vomiting. Therefore, 15 g. of butter and ghee along with *chapattis* from 1 oz. of wheat flour were used for the following experiments. The results are given in Table II :—

TABLE II.

Chylomicron counts after the ingestion of (a) 15 g. butter and (b) 15 g. ghee with chapattis from 1 oz. wheat flour.

Hours.	N. G. N.		V. N. P.		P. K. D.		K. S. R.	
	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
0	40.4	40.9	24.4	17.6	27.8	21.1	5.6	39.5
1	40.7	41.5	22.9	45.5	26.7	19.9	19.3	71.7
2	38.0	132.2	44.0	43.5	75.0	41.7	59.2	98.3
3	131.0	157.9	63.6	65.5	58.4	45.4	65.5	76.7
4	60.7	40.7	61.0	64.3	37.3	50.0	58.7	52.5
5	56.5	75.5	36.0

It can be observed from Table II that there was an increase in the particle count in about 2 to 3 hours in the case of butter as well as ghee. The increase was, however, not so marked as when 30 g. of butter were used except with the subject N. G. N. and hence the picture of the absorption process is not as clear cut as one would like it to be. The use of the smaller quantity of fat in the test meal was therefore not explored further.

DISCUSSION.

From Graphs 4 to 10 it will be clear that there exists a difference in the rates of absorption of butter-fat and coconut oil on the one hand and ground-nut and sesame oils on the other. The former are absorbed rapidly, the maximum count being reached within 2 to 3 hours. Ground-nut oil is absorbed slowly, the maximum count being reached after 4 hours. Sesame oil has given two low maxima, it seems that it is also being absorbed slowly at about the same rate as the ground-nut oil as the maximum count appeared within 3 to 5 hours.

If the chemical composition of these substances be considered, it might be possible to explain the differences found in the comparative rates of absorption. It has been observed that the lower saturated fatty acids of milk-fats, although collectively not so prominent as either oleic or palmitic acid, are the constituents which qualitatively differentiate most milk-fats from all other fats (Hilditch, 1940). In milk-fat the acids from butyric to capric (C_4 to C_{10}) account for about 8 to 9.5 per cent of the total fatty acids.

Whereas ground-nut oil and sesame oil do not contain any of the lower saturated fatty acids, coconut oil contains about 67 to 68 per cent of the acids from C_4 to C_{12} and is absorbed as rapidly as butter-fat which, however, contains only about 8 per cent of these acids.

In India, butter-fat is mainly consumed in the form of ghee. In the preparation of ghee from butter the latter is heated over the open fire till the water in the butter is completely driven off. It was natural enough to investigate the absorption of ghee in order to find out whether the heating which usually lasts about 30 minutes affected the rate of absorption. As the chylomicrographs (Graphs 4 and 5) show, ghee was absorbed at the same rate as butter.

The next step was to investigate the effect of frying on the rate of absorption of ghee. During the experiment described earlier ghee attained the temperature of 240°C . to 250°C . where it remained during the 10 minutes required for frying *purees* made out of the dough from 2 oz. of wheat flour. Here again the results show that frying had only a slight influence on the rate of appearance of fat in the blood. It is possible, however, that when ghee is kept at such a high temperature for a longer period while frying larger quantities than that tried in this experiment, the absorption of ghee-fat may be further delayed.

It has already been mentioned that the main difference between ground-nut and sesame oils on the one hand and coconut oil and butter-fat on the other is that the former do not contain fatty acids of low molecular weight, but that they contain 9 to 15 per cent of saturated C_{16} and C_{18} and 85 to 88 per cent unsaturated fatty acids of the same carbon length. It is this complete absence of fatty acids with small carbon chain that might possibly be responsible for the slower absorption of the ground-nut and sesame oils. It appears likely that the presence of short-chain fatty acids in the glyceride molecule influences the absorption of long-chain fatty acids, otherwise it would be difficult to explain the observation on the rates of absorption of coconut oil and butter-fat. In the former, as has already been pointed out, more than half of its total fatty acid content exists in the form of fatty acids up to C_{12} , whereas in butter-fat only about 8 per cent of fatty acids are up to C_{10} , and yet both fats are absorbed at about the same rate. That the degree of unsaturation is not highly important can be seen by the fact that coconut oil having an iodine value of 8 and butter-fat an iodine value of 35, were absorbed at the same rate. Then again, the ground-nut oil with an iodine value of 92 to 98 was absorbed at about the same rate as its partially hydrogenated product with an iodine value of 50. It is not suggested, however, that a completely hydrogenated product, which must have a much higher melting point, would be absorbed at the same rate as the original oil. Since the experiment with a fully hydrogenated oil was not attempted the question may be considered open.

SUMMARY.

1. The rates of absorption of butter, ghee, ground-nut oil, partially hydrogenated ground-nut oil, coconut oil and sesame oil from the intestines of healthy human subjects were determined by Frazer's 'chylomicron' technique.

2. Butter, ghee and coconut oil were found to be rapidly absorbed, whereas ground-nut oil and sesame oil were absorbed slowly.

3. Partially hydrogenated ground-nut oil with an iodine value of 50 was absorbed at approximately the same rate as the refined ground-nut oil of iodine value 98.

4. Ghee attained the temperature of 240°C. to 250°C. during frying of *purees*. When such *purees* containing 1 oz. of ghee were eaten, the latter was absorbed at a rate only slightly slower than unheated ghee.

5. It is suggested that the rate of absorption of fat from the intestines is determined among other things by the presence of fatty acids of low molecular weight constituting the fat. Quite a considerable alteration in the iodine value of fat by hydrogenation exerted little influence on its rate of absorption.

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OBSERVATIONS ON THE CAROTENOID PIGMENTS OF LOCAL VARIETIES OF YELLOW MAIZE.

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THE carotenoid pigments of yellow maize have attracted considerable interest since the investigations of Steenbock (1919) who demonstrated that yellow maize was a good source of vitamin A. Euler *et al.* (1928) stated that the activity of yellow corn was due to carotenes. Karrer *et al.* (1929) isolated zeaxanthin, the principal pigment of yellow maize, and showed it to be completely devoid of growth-promoting activity when fed to rats. Kuhn and Grundman (1934) later isolated from yellow corn cryptoxanthin and showed that most of the growth-promoting activity of yellow corn was due to this pigment. More recently Clark and Gring (1937) and Buxton (1939) have published data on the carotenoid contents of yellow corn. The latter author examined five commercial samples of yellow corn and found them to contain both carotene and cryptoxanthin, the quantities of which varied considerably in the samples examined. Fraps and Kemmerer (1941) isolated β -carotene, neo-cryptoxanthin (maxima in hexane 441μ), and another pigment which he called κ -carotene (maxima in hexane 425μ and 397μ) in addition to β -carotene and cryptoxanthin from yellow maize. All the five pigments, namely β -carotene, cryptoxanthin, neo-cryptoxanthin, κ -carotene and α -carotene, showed different degrees of vitamin A activity.

It would appear, therefore, from the literature that zeaxanthin, the principal pigment of yellow corn, is inactive; yellow corn contains several other carotenoid pigments, five of which may be physiologically active as a source of vitamin A. There are a number of varieties of yellow corn produced in this country. An investigation of their carotenoids would not only be of theoretical interest, but is of importance in the assessment of their vitamin A activity since yellow maize enters very widely into the dietaries of the people. In this paper a study of the nature of the carotenoid pigments present in fifteen local varieties of yellow corn is reported and their nutritional significance assessed.

EXPERIMENTAL.

The methods for the determination of carotene and other carotenoid pigments in plant materials are based on the discovery of Borodin (1883) that the carotenoid pigments could be separated into alcohol-soluble (hypophasic) and petroleum-ether-soluble (epiphasic) fractions. Based upon this discovery Willstätter and Stoll (1913) developed a method for the colorimetric estimation of these pigments which has almost universally been adopted by other investigators in this field. The method has, however, been modified and improved in recent years. The modified method also depends upon pigment distribution between alcohol (90 to 92 per cent CH_3OH) and petroleum ether for the separation of carotene and xanthophyll (Schertz, 1928; Guilbert, 1934; Peterson *et al.*, 1937). A number of the other workers have pointed out discrepancies in the method even when improvements suggested are taken into consideration. It has been found that the non-carotene chromogens, which are inactive, may appear in the epiphasic layer (Virtanen, 1933; Hartman *et al.*, 1934; Peterson *et al.*, 1935, 1937a; Kane *et al.*, 1936; Hayden *et al.*, 1937; Quackenbush, Steenbock and Peterson, 1938; Whitnah *et al.*, 1939). The inactive isomer, lycopene, is

also estimated as carotene by the use of this technique. A certain amount of xanthophyll itself may remain in the petroleum ether layer and the method based upon phase partition can estimate only the gross carotene content without taking into account the constituent pigments.

A method based on fractional adsorption since called chromatographic separation was first devised by Tswett (1906). This method has been found very helpful in the separation of different carotenoid pigments. The method has been developed more recently by a number of investigators (Palmer and Eckles, 1914; Kuhn *et al.*, 1931; Kuhn and Brockmann, 1932) and principally by Zechmeister and Chlcnoky (1941) and is universally followed in all modern works on carotenoid pigments. The chromatographic separation can be used alone or combined with phase partition. A combination of these methods results in a successful separation of the different pigments. In this investigation both these techniques have been followed.

The procedure for complete extraction of the carotenoid pigments followed in this investigation was that described in the handbook of Association of Official Agricultural Chemists (1940). It involved the refluxing of about 20 g. of finely ground yellow corn with 200 c.c. of freshly prepared 12 per cent methanolic potassium hydroxide. The alcoholic extract so obtained, as well as the residue, was extracted completely with petroleum ether. The petroleum-ether fraction after washing away of alkali represents the entire carotenoid pigments of the original sample. Zeaxanthin could be separated from this fraction by repeated extractions with 90 to 92 per cent CH_3OH . The methanolic fraction is then distilled off under partial vacuum in an inert atmosphere and the dissolved pigments taken up in petroleum ether. This results in two fractions which are separately chromatographed.

The selection of a suitable adsorbant is of considerable importance in successful chromatographic separation. Calcium oxide, magnesium oxide and aluminium oxide which are commonly used in carotenoid separation were tried. In our investigations calcium oxide was found to be not a very satisfactory adsorbant. Magnesium oxide gave good results, but when this was used, the movement of the liquid through the column was slow and there was a greater tendency for the pigments to get oxidized. Aluminium oxide gave the most satisfactory results. Several brands of aluminium oxide were tried with varying results. Aluminium oxide prepared in the laboratory from aluminium sulphate gave fairly good results on activation. Another brand (Hirasawa Pharmaceutical Laboratory, Tokyo, Japan) was found very satisfactory when activated by heating at 200°C . in a current of CO_2 for two hours. This brand was, therefore, used after activation in all experiments described in this investigation.

In chromatographic analysis certain precautions are of importance. The tube containing the adsorbant should be very evenly packed to allow a steady and even flow of the liquid through the column. The upper surface of the column should remain constantly covered with the liquid throughout the process. It is necessary that the petroleum ether extract to be chromatographed should be free from alcohol as otherwise the pigments are not properly adsorbed. The adsorbant should have proper activity in relation to the nature and quantity of the pigments to be separated. Too strong or too weak an adsorbant leads to inaccurate results. An adsorbant which is highly activated may hold the pigments too tenaciously for being eluted fully, while one, which is less activated, is apt to have a poor power of selective adsorption necessary for the separation of closely related pigments.

The solvent used for chromatographic separation in this study was petroleum ether (40°C . to 60°C .) distilled from ordinary petrol. Elution was effected by petroleum ether containing 1 per cent ethanol.

After elution the amount of different pigments were determined colorimetrically matching the solutions against 0.04 per cent $\text{K}_2\text{Cr}_2\text{O}_7$ solution in a Bio-Klett colorimeter. The amount of the pigments was determined by reference to a standard curve made by comparing different concentrations of pure β -carotene with 0.04 per cent $\text{K}_2\text{Cr}_2\text{O}_7$ solution. The

same curve was used for the estimation of all the different carotenoid pigments examined. An atmosphere of CO₂ was used, wherever possible, to prevent oxidation and the pigments were always protected from exposure to light.

Recovery of the pigments from the chromatogram was not found to be quantitative due largely to incomplete elution from the adsorbant. Experiments were conducted to determine the loss which occurs in this process. The data of these experiments are summarized in Table I:—

TABLE I.

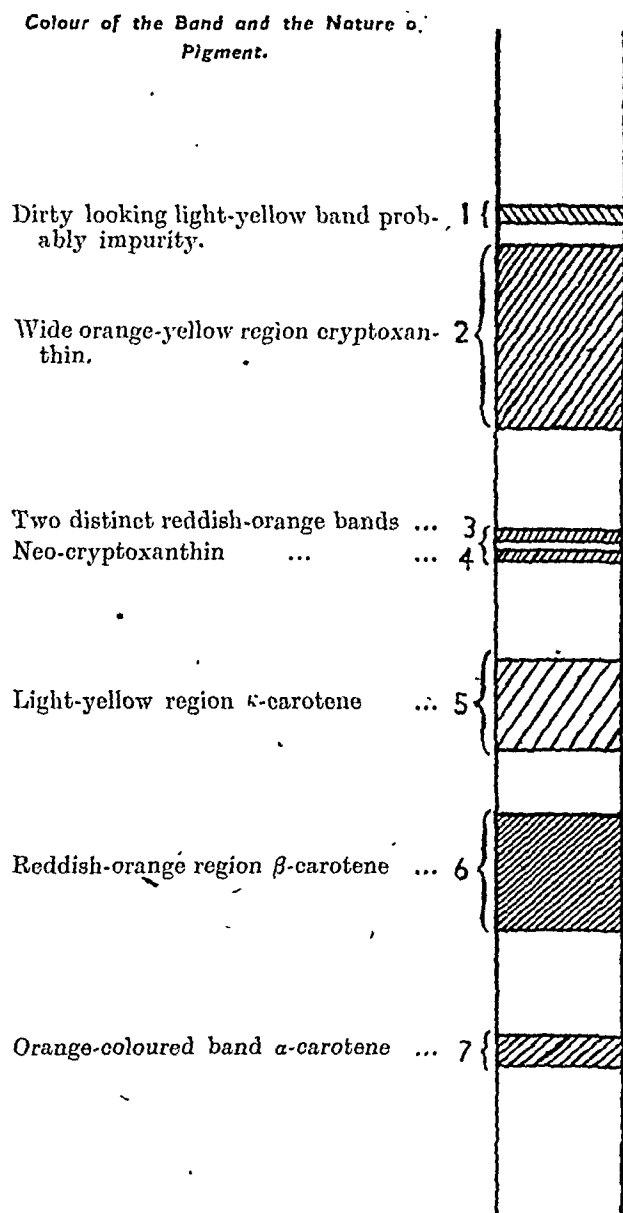
Number.	Colour of the sample.		Total pigments found before chromatographic adsorption (μ g. per g.).	Total pigments found by chromatographic adsorption (μ g. per g.).	Loss per cent.
1.	Very light yellow	...	1.98	1.90	4.0
2.	Medium yellow	...	9.84	8.93	9.2
3.	Dark yellow	...	43.86	37.96	13.4
4.	Dark red	...	44.87	41.12	8.3
5.	Medium yellow	...	12.11	10.96	9.5
6.	Deep yellow	...	23.83	20.43	14.2
7.	Medium yellow	...	19.43	18.03	7.2
8.	Deep yellow	...	31.2	28.12	9.8

Table I shows that loss of the pigment during chromatographic separation may vary between 4 and 15 per cent. Loss of the pigment during chromatography appeared to be related to the amount of zeaxanthin present. This pigment showed greater degree of incomplete elution than the hydrocarbon pigments present.

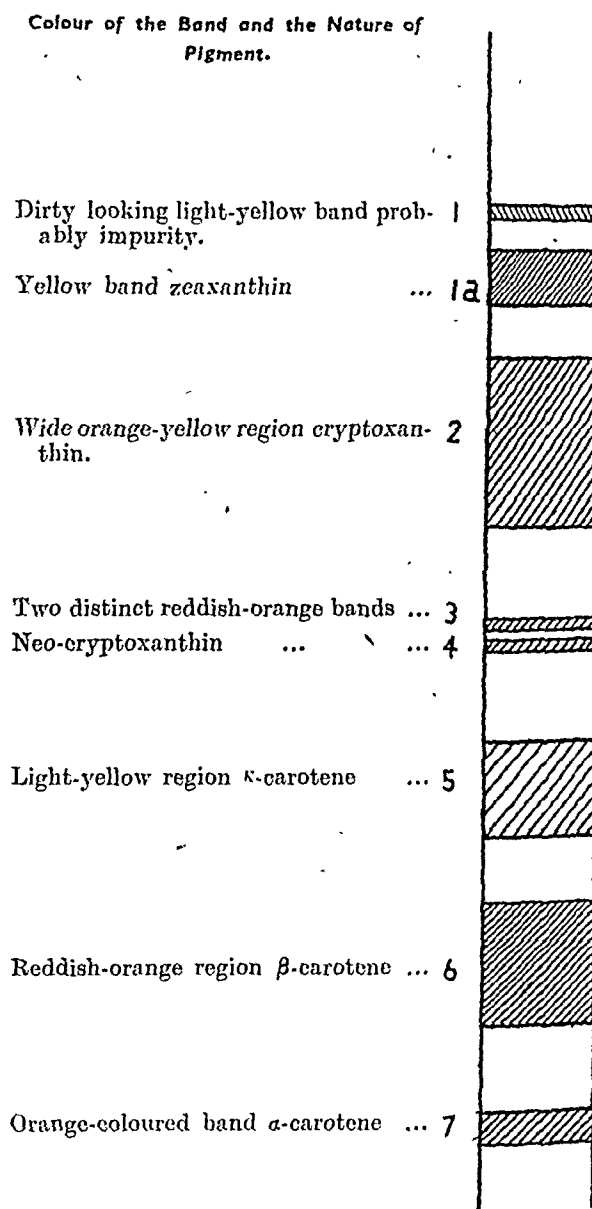
Fifteen varieties of maize grown in different parts of Punjab were investigated.

With the exception of white and very light-yellow varieties, all the rest of the samples showed the presence of 7 to 8 distinct bands in the chromatogram depending upon whether zeaxanthin had been separated earlier by the phase separation (*see* Chromatographs 1 and 2).

1. It was a dirty looking light-yellow narrow band from which only traces of pigments could be eluted out. Probably it represents impurities in the solution.



CHROMATOGRAPH 1.—After phase separation.



CHROMATOGRAPH 2.—Without phase separation.

1a. It was a yellow band. The pigment was extracted and identified as zeaxanthin by its absorption spectra.

2. This was a wide orange-yellow region. The pigment was extracted and identified as cryptoxanthin by its absorption spectra.

3 and 4. These were two distinct reddish-orange bands in very close proximity. Zechmeister and Tuzson (1938) noticed a band close to cryptoxanthin in their chromatograms, and described it as an isomerization product of cryptoxanthin and named it neo-cryptoxanthin. Fraps and Kemmerer (*loc. cit.*) noticed three bands, one orange-coloured band identical with neo-cryptoxanthin and very close to it two reddish-orange bands which could not be separated from the other and estimated as neo-cryptoxanthin. We have not been able to isolate these pigments. They most probably represent isomers of cryptoxanthin and have, therefore, been estimated as neo-cryptoxanthin.

5. This was a light-yellow region. It is a new pigment named α -carotene (Fraps and Kemmerer, *loc. cit.*). It is biologically active.

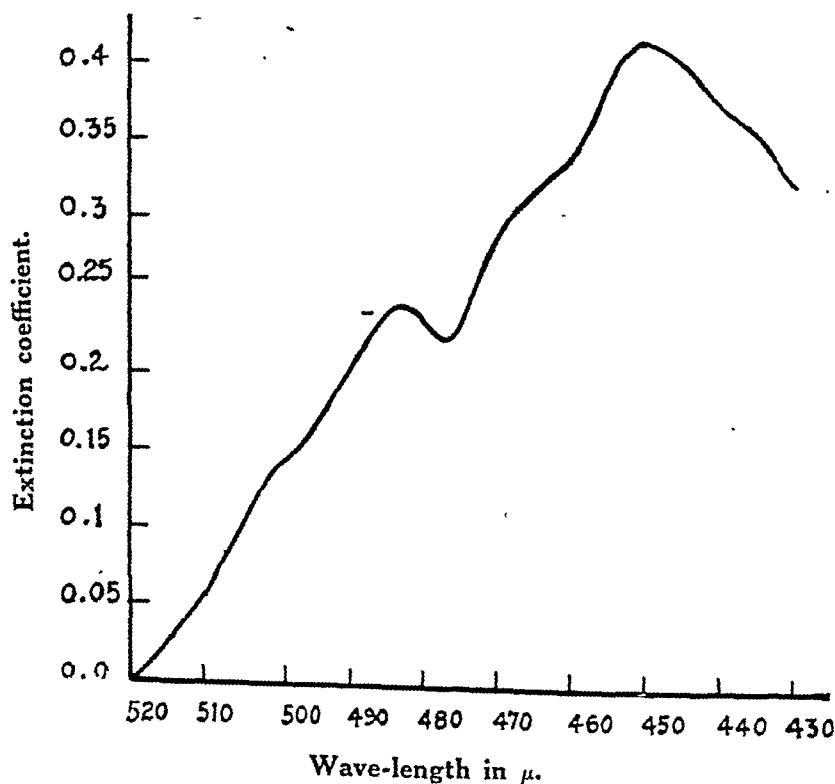
6. It was a reddish-orange region. The pigment was isolated and identified as β -carotene from its absorption spectra.

7. It was an orange-coloured band and identified as α -carotene by its absorption spectra and optical activity.

For the study of the physical properties of these pigments, namely, absorption spectra and optical activity, the pigments were purified by rechromatographing them twice. The absorption spectra of the purified pigments was studied in two solvents, petroleum ether (B.P. 40°C. to 60°C.) and ethyl alcohol, using a Nutting's photometer with Adam-Hilger spectrometer and a pointolite lamp of 100 C.P. as the source of light.

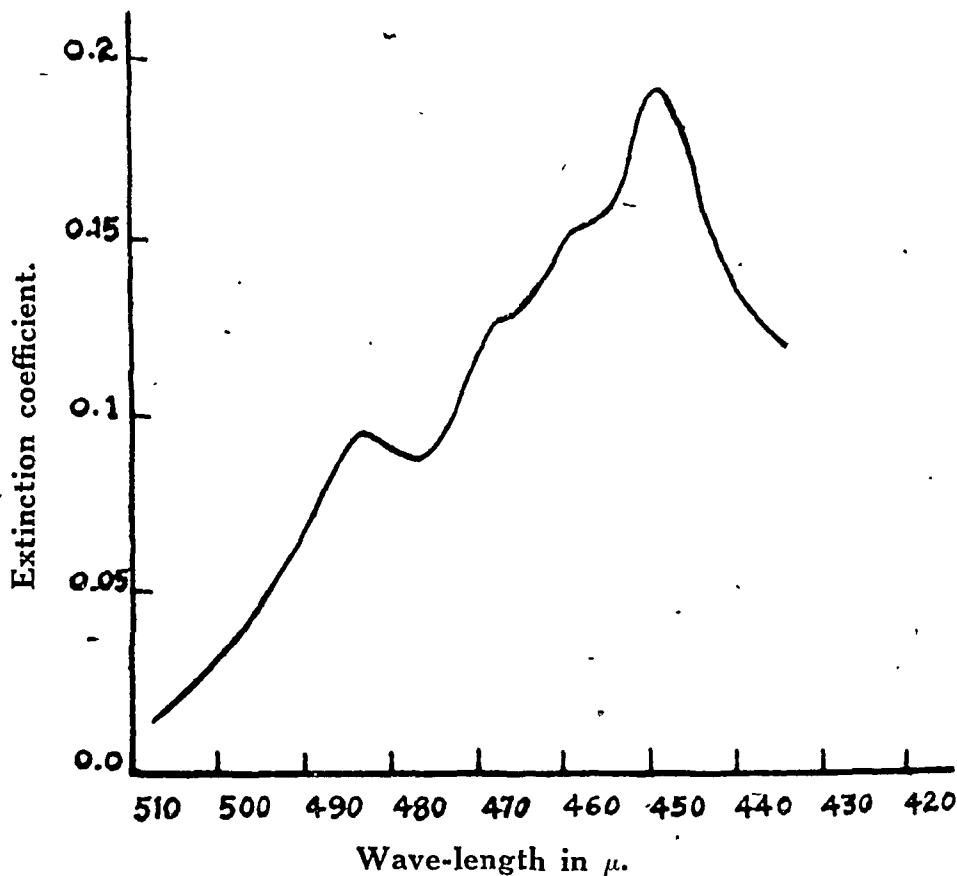
Typical absorption curves for the three pigments are shown in Graphs 1, 2 and 3. They show absorption bands in the following regions:—

Cryptoxanthin	484 μ .	451 μ .
Zeaxanthin	483 μ .	451 μ .
β -carotene	482 μ .	452 μ .

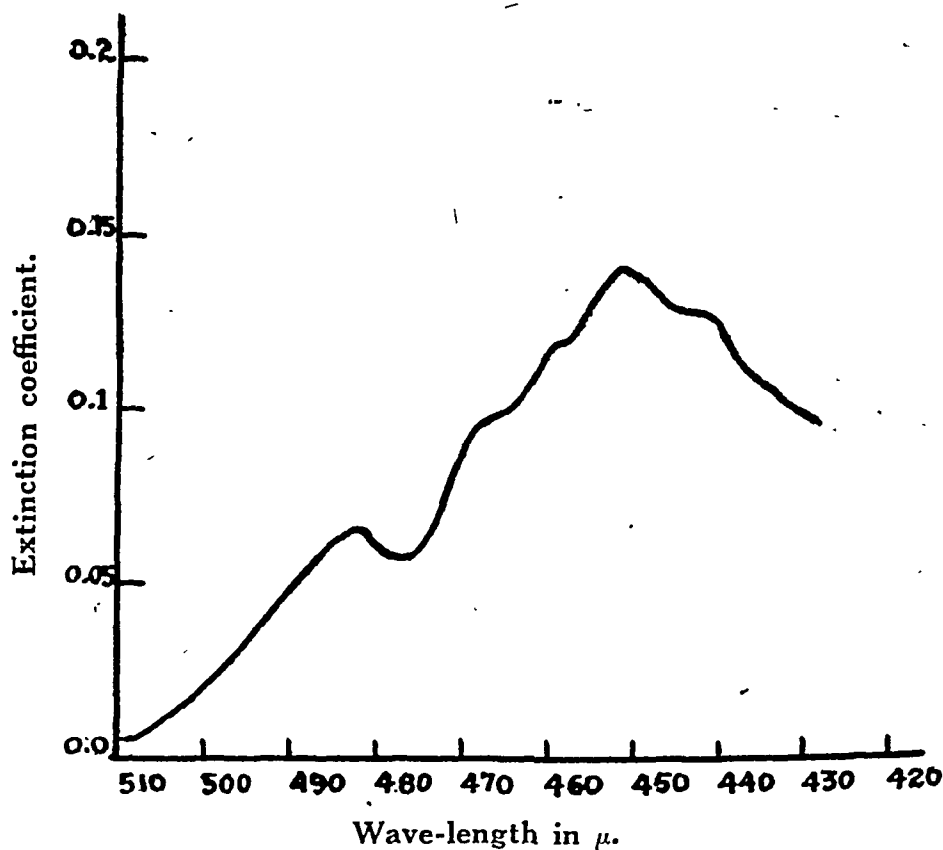


GRAPH 1.—Zeaxanthin in ethyl alcohol.

Results of analyses are shown in Tables II and III. Table II shows the quantity of different pigments present per gramme of the sample of corn, while Table III shows the percentages of different pigments present.



GRAPH 2.—Cryptoxanthin in petroleum ether (B.P. 40°C. to 60°C.).



GRAPH 3.— β -carotené in petroleum ether (B.P. 40°C. to 60°C.).

TABLE II.

Different carotenoid pigments of yellow corn.

Number.	Place of production.	Colour.	Zeaxanthin μg./g.	Cryptoxanthin μg./g.	Neo- cryptoxanthin μg./g.	κ-carotene μg./g.	β-carotene μg./g.	α-carotene μg./g.	Total pigments μg./g.	Total vitamin A potency I.U. per g.
1.	Ferozepur district	0.16*	...
2.	Lahore district	0.17*	...
3.	Lahore district	...	Very light yellow	1.24	0.49	0.00	0.17	0.00	1.90	0.69
4.	Lahore district	...	Yellow	25.50	5.45	0.34	2.74	0.36	34.82	10.05
5.	Jullundur district	...	Yellow	29.40	6.28	0.55	3.72	0.51	40.78	12.58
6.	Jullundur district	...	Dark yellow	28.70	6.11	0.46	2.24	0.51	38.66	10.17
7.	Jullundur district	...	Deep yellow	...	5.76	0.46	2.46	0.65	9.72†	10.15
8.	Nakodar tehsil	...	Deep red	28.70	7.52	0.39	2.61	0.42	40.74	12.21
9.	Nakodar tehsil	...	Deep yellow	...	7.23	1.26†	2.52	0.36	11.37†	11.58
10.	Nakodar tehsil	...	Yellow	...	6.89	1.27†	2.79	0.32	11.27†	11.72
11.	Moga area	...	Medium yellow	12.90	3.61	0.56	1.86	0.10	19.32	6.90
12.	Kangra district	...	Medium yellow	10.20	5.58	0.56†	2.18	0.24	18.76	8.85
13.	Ludhiana district	...	Dark yellow	13.20	4.10	0.30	1.95	0.19	19.92	7.23
14.	Ferozepur district	...	Medium yellow	21.30	4.48	0.33†	1.69	0.32	28.12	7.09
15.	Ferozepur district	...	Dark yellow	...	4.40	0.50†	1.35	0.19	6.44†	6.49

* Contained only traces of the yellow carotenoid pigments.

† Neo-cryptoxanthin and κ-carotene formed two separate bands but could not be separated and hence they were estimated together.

‡ Zeaxanthin was not determined. Total represents all the other pigments excepting zeaxanthin.

TABLE III.

Percentage of different carotenoid pigments of yellow corn.

Number.	Place of production.	Colour.	Total pigments μg./g.	Vitamin A active pigments μg./g.	Zeaxanthin per cent.	Cryptoxanthin per cent.	Neo- cryptoxanthin per cent.	κ-carotene per cent.	β-carotene per cent.	α-carotene per cent.
1.	Ferozepur district	White	0.16
2.	Lahore district	White	0.17
3.	Lahore district	Very light yellow	1.90	0.66	65.26	25.79	0.00	0.00	8.94	0.00
4.	Lahore district	Yellow	34.82	9.32	73.23	15.65	1.23	0.97	7.86	1.03
5.	Jullundur district	Yellow	40.78	11.38	72.09	15.39	0.78	1.34	9.12	1.25
6.	Jullundur district	Dark yellow	38.66	9.96	74.23	15.80	1.65	1.19	5.79	1.32
7.	Jullundur district	Deep yellow	*	9.72
8.	Nakodar tehsil	Deep red	40.74	12.04	70.44	18.45	2.70	0.95	6.40	1.03
9.	Nakodar tehsil	Deep yellow	*	11.37
10.	Nakodar tehsil	Yellow	*	11.27
11.	Moga area	Medium yellow	19.32	6.42	66.77	18.68	2.90	1.50	9.62	0.51
12.	Kangra district	Medium yellow	18.76	8.56	54.37	29.74	2.98†	...	11.62	1.28
13.	Ludhiana district	Dark yellow	19.92	6.72	66.26	20.58	1.50	0.90	9.78	0.95
14.	Ferozepur district	Medium yellow	28.12	6.82	75.74	15.93	1.17†	...	6.01	1.13
15.	Ferozepur district	Dark yellow	*	6.44

* Since the value of zeaxanthin was not available, the percentages could not be calculated.

† Neo-cryptoxanthin plus κ-carotene.

DISCUSSION.

In Tables II and III the results of analyses of two white, one very light-yellow, one deep-red and 11 medium to deep-yellow varieties of yellow corn are recorded. The white varieties contained only traces of the pigments which were too small to be separated by chromatographic analysis. The very light-yellow variety also showed less than 2 $\mu\text{g./g.}$ of total pigments and its chromatogram showed only two bands corresponding to cryptoxanthin and β -carotene. Only one red variety of corn was available which on analysis showed cryptoxanthin and β -carotene contents of about the same order as other yellow varieties. In the other yellow varieties which formed the bulk of the samples analysed and which constitute the greater part of the type of yellow maize produced in the province, the total pigments content varied between 18.76 $\mu\text{g./g.}$ to 40.78 $\mu\text{g./g.}$ of which as much as 10 $\mu\text{g./g.}$ to 29.4 $\mu\text{g./g.}$ was zeaxanthin representing 54.37 to 75.74 per cent of the total pigments. The cryptoxanthin content varied from 3.61 $\mu\text{g./g.}$ to 7.5 $\mu\text{g./g.}$ representing 15 to 29 per cent of the total pigments. The amount of β -carotene was only 1.35 $\mu\text{g./g.}$ to 3.72 $\mu\text{g./g.}$ representing 6 to 11 per cent of the total carotenoid. The α -carotene was only about 0.5 $\mu\text{g./g.}$ or less and did not represent more than 0.5 to 3.0 per cent of the total pigments. The quantities of neo-cryptoxanthin and κ -carotene were also of the order of 1 to 3 per cent.

The vitamin A potency of the various varieties has been calculated on the basis of 1 I.U. = 0.6 γ / β -carotene and 1.2 γ of the other pigments excepting zeaxanthin. The potency of the very dark-yellow and red varieties varied between 10 I.U./g. to 12.6 I.U./g. while the medium-yellow variety showed activity equivalent to 6.5 I.U./g. to 9.0 I.U./g.

Somewhat low values for carotene content are recorded in the literature (Myburgh, 1935; Fraps, Treichler and Kemmerer, 1936; Clark and Gring, *loc. cit.*). Records of studies in India showed only 0.42 I.U./g. to 2.8 I.U./g. (De, 1936; Ahmad, Mullick and Mazumdar, 1937; Health Bulletin No. 23, 1941). Only a few varieties reported by Shinn *et al.* (1935) and Russell *et al.* (1935) showed as much potency as 11 I.U./g. to 15 I.U./g. The vitamin A content of Punjab maize therefore is among the most potent varieties reported.

SUMMARY.

The composition of 15 different varieties of yellow corn grown in different parts of the Punjab with respect to their different carotenoid pigments has been determined by the method of chromatographic separation and colorimetric estimation. Six different pigments were detected in the samples examined which were identified as zeaxanthin, cryptoxanthin, β -carotene, α -carotene, κ -carotene, and an isomer of cryptoxanthin called neo-cryptoxanthin.

Varieties of maize varying in colour from moderate-yellow to dark-yellow and deep-red showed the values ranging from 18.76 $\mu\text{g./g.}$ to 40.78 $\mu\text{g./g.}$ for the total pigments.

From the amount of different pigments present the vitamin A potency of yellow corn has been assessed at 6.5 I.U./g. to 9.0 I.U./g. for medium-yellow varieties, and 10 I.U./g. to 12.5 I.U./g. for the dark-yellow to the deep-red varieties.

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OBSERVATIONS ON THE CAROTENOID PIGMENTS OF THE MANGO FRUIT.

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VERY few investigations have been carried out on the lipochrome pigments of the mango which is one of the commonest fruits in India and is exceedingly rich in these pigments. The studies so far recorded include a few observations on its total content of hydrocarbon pigments (Ahmad *et al.*, 1937; De, 1936, 1937) and an observation on the increase of carotenoid pigments during ripening (Ramasarma and Banerjee, 1940). The first attempt to isolate the carotenoid pigments of the mango was made by three Japanese workers (Yamamoto *et al.*, 1932) who obtained a crystalline preparation which on the basis of its optical activity and absorption spectra was described as a mixture of α - and β -carotenes. More recently, Ramasarma, Hakim and Rao (1943) investigated the carotenoid pigments of the Badami mango showing them to consist of xanthophyll esters with a trace of free xanthophyll and β -carotene.

In view of the abundance in which the mango fruit occurs in India, and in view of the fact that it enters into the dietaries of all sections of the population during its season, while at the same time it is a rich source of carotene, its importance for a people whose diets are generally deficient in vitamin A cannot be minimized. The carotenoid pigments of different varieties of this fruit have therefore been investigated in detail and their exact potency as a source of vitamin A has been assessed. Ten different varieties of mangoes available in Delhi market during the summer months of June, July and August were studied.

EXPERIMENTAL.

The procedure followed for the extraction of the total carotenoid pigments was that of Association of Official Agricultural Chemists (1940). Specially activated alumina, described in an earlier paper (Sadana and Ahmad, 1946), was used as the adsorbant. A concentrated solution of the pigments in petroleum ether was used for chromatographic analysis and the bands were developed by petroleum ether containing 10 per cent benzene. The various pigments were eluted by petroleum ether containing 2 per cent ethyl alcohol, and were estimated colorimetrically by means of a standard curve of β -carotene against 0.04 per cent $K_2Cr_2O_7$. The results are expressed as $\mu g./g.$ present in terms of β -carotene.

The following varieties of mangoes which were available in the Delhi market during the summer months were investigated:—

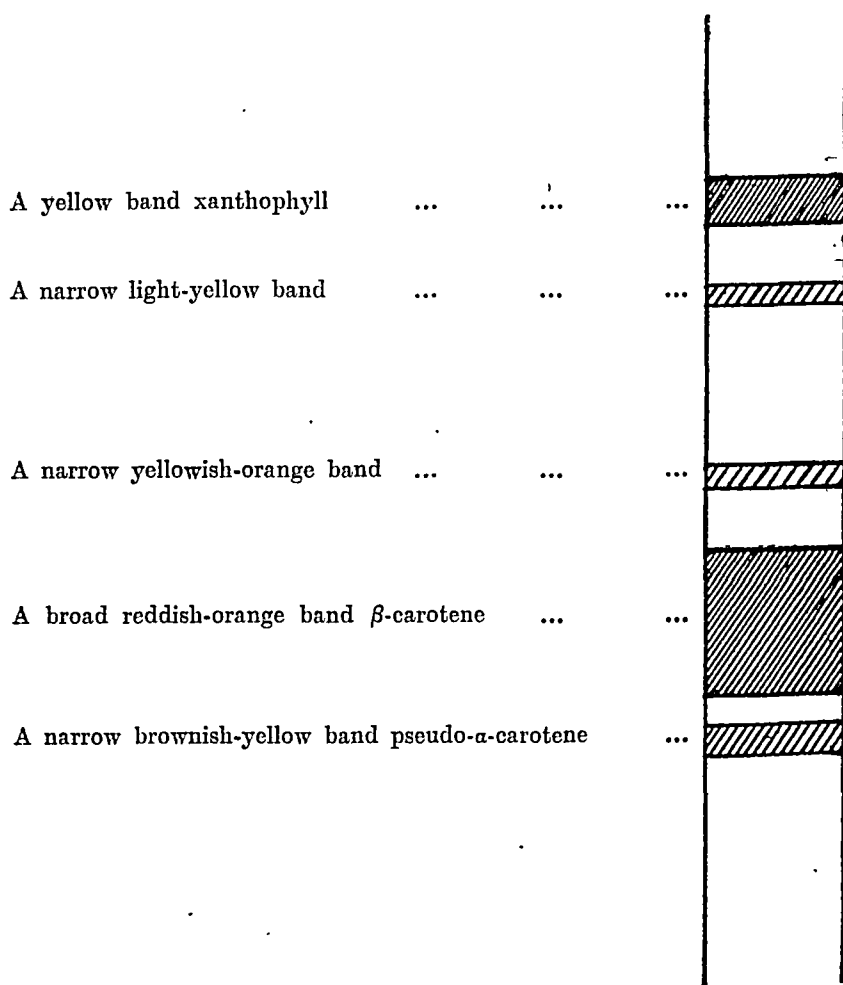
1. Madras mango.
2. Benares Langra.
3. Lucknow Dasherri.
4. Delhi Desi variety—
 - (a) Large size.
 - (b) Small size.
5. Calcutta Fazli mango.
6. Calcutta Langra.
7. Ratol (a) Desi variety.
8. Ratol (b) Superior variety.
9. Tammoria or Saffron.

10. Chounsa or Samar Bahisht—

- (a) Unripe.
- (b) Partially ripe.
- (c) Fully ripe.

Chromatograms of all these varieties, except Madras mango, showed five distinct bands (see Chromatograph) which are characterized as follows :—

- (1) This was a yellow band. It represents xanthophyll as shown by phase separation.
- (2) This was a narrow light-yellow band. The pigment has not been identified.
- (3) This was again a narrow yellowish-orange band and the pigment has not been identified. Both pigments 2 and 3 appear to be of a hydrocarbon nature since they are present in the same intensity even after xanthophyll has been removed by repeated extraction with methyl alcohol. It is probably identical with carotenoid α of Kemmerer and Fraps (1943).
- (4) This was a broad reddish-orange band. This represents β -carotene, as identified by its absorption spectra.
- (5) This was a brownish-yellow band. It is presumably an isomerized product of β -carotene, called by Zechmeister and Tuzson (1938), and Gillam, el Ridi and Kons (1937) as pseudo- α -carotene. It is biologically as potent as β -carotene and its absorption spectra is similar to that of α -carotene (Gillam, el Ridi and Kons, *loc. cit.*; Gillam and el Ridi, 1936; Mann, 1944). Kemmerer and Fraps (*loc. cit.*) claim that its activity is only half that of β -carotene.



CHROMATOGRAPH.—Typical chromatogram of the pigments of the mango fruit,

There is invariably some loss in the pigments during the process of chromatographic analysis in spite of the fact that all precautions were taken to protect the pigments from light, oxidation and any undue standing of the solutions was avoided. This loss has been estimated in each case and is recorded in Table I. In Table II the analytical data are summarized. In all cases, two independent analyses were made of each sample and when the results were not concordant, a third analysis was made. In this table the average figures are shown.

TABLE I.

Percentage loss of the pigments during chromatographic analysis.

1.	2.	3.	4.	5.
Number.	Variety.	Total pigments found before chromatographic adsorption ($\mu\text{g. per g.}$).	Total of individual pigments obtained after chromatographic separation ($\mu\text{g. per g.}$).	Loss, per cent.
1	Madras ...	16.83	13.79	18.0
2	Benares Langra ...	62.29	59.65	4.2
3	Lucknow Dasherri ...	53.08	50.13	5.5
4	Delhi Desi—Large size ...	23.75	22.41	5.6
	„ Small size ...	14.46	13.11	9.3
5	Calcutta Fazli ...	47.92	42.58	11.1
6	Calcutta Langra ...	30.34	28.72	5.3
7	Ratol, Desi variety ...	34.38	31.35	8.7
8	Ratol, Superior variety ...	66.13	62.34	5.7
9	Tammoria or Saffron ...	64.39	61.79	4.0
10	Chounsa—Unripe ...	4.37	3.60	17.6
	„ Fully ripe ...	26.96	24.05	10.7

The figures shown in column 4 were obtained by adding the values obtained for each individual constituent after chromatographic analysis. The colorimetric estimations were made only against a standard curve of β -carotene. It cannot be affirmed with certainty that figures for percentage loss represent a true loss. They are, however, suggestive and indicate the order of losses involved in the use of the technique. The loss varies generally from 4.0 per cent to 18.0 per cent.

TABLE II.

Different carotenoid pigments of the mango fruit and their vitamin A activity.

Number.	Variety.	Xanthophyll.		Unidentified pigments. (Band II.)		Unidentified pigments. (Band III.)		β -carotene.		Pseudo- α -carotene.		Total active pigments.		Vitamin-A potency.
		$\mu\text{g./g.}$	Per cent of total pigments.	$\mu\text{g./g.}$	Per cent.	$\mu\text{g./g.}$	Per cent.	$\mu\text{g./g.}$	Per cent.	$\mu\text{g./g.}$	Per cent.	$\mu\text{g./g.}$	Per cent.	
1	Madras ...	13.79	5.21	37.8	0.0	0.0	0.0	8.58	62.2	0.0	0.0	8.58	62.2	14.3
2	Benares (Langra) ...	59.65	39.06	65.48	0.93	0.71	1.19	18.31	30.70	1.01	1.7	19.32	32.4	32.2
3	Lucknow (Dasher) ...	50.13	18.92†	37.7†	†	0.39	0.78	29.09	58.02	1.73	3.4	30.82	61.42	51.5
4	Delhi (Desi quality)—													
	(a) Large size ...	22.41	15.44	68.89	1.2	0.32	1.4	5.84	26.06	0.54	2.4	6.38	28.46	10.6
	(b) Small size ...	13.11	7.92	60.4	1.8	0.0	0.0	4.77	36.38	0.18	1.37	4.95	37.75	8.2
5	Calcutta (Fazli) ...	42.58	13.91	32.66	0.75	1.17	2.74	24.58	57.7	2.6	6.1	27.18	63.8	45.3
6	Calcutta (Langra) ...	28.72	14.01	48.78	1.04	0.0	0.0	13.85	48.2	0.56	1.95	14.41	50.15	24.0
7	Ratol (Desi quality) ...	31.35	11.67	37.2	0.73	0.70	2.2	17.46	55.69	1.29	4.1	18.75	59.8	31.2
8	Ratol (Superior quality) ...	62.34	20.23	32.45	0.75	1.16	1.8	39.01	64.0	0.57	0.9	40.48	64.9	67.4
9	Tammoria or Saffron ...	61.79	33.26	53.8	0.79	0.54	0.87	26.38	42.7	1.12	1.8	27.50	44.5	45.8
10	Chounsa—													
	(a) Unripe ...	3.6	1.87	51.9	0.0	0.0	0.0	1.65	45.8	0.08	2.22	1.73	48.02	2.88
	(b) Partially ripe ...	13.91	7.85	56.4	0.0	0.52	3.7	4.77	34.29	0.77	5.5	5.54	39.8	9.2
	(c) Fully ripe ...	24.05	14.45	60.08	0.0	*	*	9.08*	37.7*	0.52	2.16	9.6	39.9	16.0

* β -carotene + Band III.

† Xanthophyll + Band II.

CONCLUSIONS.

The result of chromatographic separation of the carotenoid pigments of different varieties of mango fruits, summarized in Table II, shows the presence of distinctly five different pigments, the principal ones being xanthophyll and β -carotene. The quantities of β -carotene and xanthophyll present generally vary between 40 to 60 per cent. The other three pigments are present in relatively small proportions, one of these is presumably pseudo- α -carotene, an isomer of β -carotene and occurs very close to β -carotene in the chromatogram. This has been found to vary from 0.0 to 5.5 per cent. The other two pigments whose identity has not been established but are likely to be of a hydrocarbon nature occur only in relatively small proportions. Their fractional adsorbability and the position of the bands in the chromatogram does not indicate that any of these is α -carotene, the presence of which in the mango was indicated by the Japanese investigators Yamamoto *et al.* (*loc. cit.*). The total quantity of both these pigments is exceedingly small and does not go beyond 1 to 3 per cent.

There appears to be a considerable variation in the carotenoid content of the different varieties, the range being 30 $\mu\text{g./g.}$ to 62 $\mu\text{g./g.}$ Benares Langra, Lucknow Dasher, superior quality Ratol and Saffron are among the rich varieties, while Madras and ordinary local Desi varieties show the lowest amount. β -carotene content in the same proportions shows variability, the richer varieties showing 18 $\mu\text{g./g.}$ to 40 $\mu\text{g./g.}$ and the poorer ones from 5 $\mu\text{g./g.}$ to 8 $\mu\text{g./g.}$

The vitamin A potency shows a variation range from 8 to 69 I.U. per g. In the richer varieties, the figures lie between 32 to 67 I.U. per g. Earlier investigations have shown values as high as 26 to 90 I.U. per g. (De, 1937) and 46 to 128 I.U. per g. (Ahmad *et al.*, *loc. cit.*) in the different varieties examined by them. These results are obviously high because they are based on the total content of the carotenoid pigments obtained in the epiphasic layer. Our estimates are based only on quantity of β -carotene and its isomer, pseudo- α -carotene.

SUMMARY.

The carotenoid content of ten different varieties of mangoes available in Delhi market has been determined by the method of chromatographic separation and colorimetric estimation. Five different pigments have been detected in almost all the varieties examined. Three pigments have been identified, namely xanthophyll, β -carotene and pseudo- α -carotene. The two other pigments have not been identified. These two pigments also appear to be of hydrocarbon nature.

The various varieties examined showed a total carotenoid content ranging from 30 $\mu\text{g.}$ to 60 $\mu\text{g.}$ per g. of which the active pigments contribute 5 $\mu\text{g.}$ to 40 $\mu\text{g.}$ per g. of the total pigments.

The vitamin A potency has been assessed at 8 to 16 I.U. per g. for poor varieties and 32 to 67 I.U. per g. for rich varieties.

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OBSERVATIONS ON THE VITAMIN A CONTENT OF BUFFALO BUTTER-FAT (GHEE).

EFFECT OF THE METHOD OF PREPARATION, STORAGE AND COOKING.

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INTRODUCTION.

VITAMIN A occurs in appreciable quantities in the milk-fat, which constitutes one of the most important sources of this factor in common human dietaries. Ghee or clarified butter of both cows and buffaloes, preferred on account of its better keeping qualities, is consumed considerably in India and hence its vitamin A content is of considerable interest.

Some investigations have been undertaken in India on the vitamin A content of milk and butter (De, 1937; Ray, Karamchand and Govind Rao, 1941; Sen and Rai Sarkar, 1942). These are, however, largely confined to the products obtained from the cow. Buffalo milk and butter which are equally popular have so far received little attention. The authors have undertaken a systematic study of the vitamin A content of buffalo milk-fat.

Earlier reports in the literature indicate a somewhat lower vitamin A content in ghee as compared to butter-fat obtained in other countries (De, *loc. cit.*). These observations raise the important question whether the butter-fat as excreted in the milk by Indian cows is itself poorer in vitamin A, or whether there is destruction of vitamin A later in the process of the preparation of ghee from the milk. The process common in India is essentially different from those used in other countries. Therefore, a careful study of the effect of the method of preparation of ghee from milk on vitamin A content has also been made.

In India ghee is sometimes stored for long intervals before it is consumed. It was, therefore, desirable to investigate whether the vitamin A content is lost to any appreciable extent during the period of storage. At the same time the effect of the common method of cooking upon the vitamin A content of ghee has been determined. It may be mentioned here that buffalo ghee is practically devoid of carotene, therefore, the study has been confined to the estimation of vitamin A alone.

EXPERIMENTAL.

The spectrographic method was used for the assay of vitamin A. Morton (1940) who has undertaken extensive investigations on the spectrographic assay of vitamin A has remarked:—

"Provided that the absorption spectrum of a natural product shows normal vitamin A band with good persistence it affords a better estimate of potency than almost any biological assay which is normally possible."

At first the method tried consisted of direct spectrographic determination of vitamin A in the whole butter-fat and using as control the de-vitaminized fat from which the vitamin had been removed with the help of suitable adsorbants. Shrewsberry and Kraybill (1933) and Kraybill and Shrewsberry (1936) have shown that both activated charcoal and Lloyd's reagent remove practically all vitamin A and carotene material from butter-fat. We found that these adsorbants also removed some of the non-vitamin constituents from the fat. The controls gave low adsorption values with the result that higher values for vitamin A for the samples were obtained. This method was, therefore, discarded. Neal, Haurand and Luckman (1941) have stated that these objections can be removed by working with the

unsaponifiable fraction of the butter-fat. In view of this the problem resolved itself into three phases : (i) development of a satisfactory technique for the extraction of the unsaponifiable material containing all the vitamin A present in the original sample, (ii) selection of a satisfactory solvent for the unsaponifiable portions, and (iii) obtaining a control material containing all the constituents of the unsaponifiable material of the particular sample excepting vitamin A. Neal *et al.* (*loc. cit.*) found that vitamin A and carotene could be successfully destroyed by ultra-violet irradiation of a cyclohexane solution of the unsaponifiable fraction of the sample of butter-fat. This solution serves as a very satisfactory control for the spectrophotometric measurements of vitamin A. We, therefore, based our method of estimation on the technique developed by Neal *et al.* (*loc. cit.*). The extraction of non-saponifiable fractions was, however, carried out according to the method used by Jones and Haines (1943). The procedure is very briefly described below :—

Saponification and extraction of the non-saponifiable fraction.—About 5 g. of ghee were weighed into a 250 ml. ground-glass joint flask, fitted with an air condenser. 1.25 ml. of 56 per cent solution of KOH and 10 ml. alcohol (at least 25 per cent) were added for every 1 g. of fat weighed. The mixture was boiled on a sand-bath for 20 minutes. After cooling to 30°C. or 40°C., 180 ml. of water (approximately 4 times the quantity of alcohol used) was added to the mixture. The mixture was ice-cooled and extracted 4 times with 70 ml., 40 ml., 30 ml., and 30 ml. portions of peroxide-free di-ethyl ether. The extracts were mixed and washed successively with 160 ml. ice-cold water, 125 ml. to 150 ml. ice-cold N. NaOH solution, and twice with ice-cold water to wash the extract free of alkali. The ether was distilled off in an atmosphere of carbon dioxide and the residue was dried by once evaporation with alcohol. The dried residue was made to 25 ml. with absolute ethyl alcohol and examined spectrographically.

Preparation of the control solution.—Ten ml. of the above solution were taken in a 4-inch wide porcelain dish and placed at a distance of 10 cm. from the radiating tube of the mercury ultra-violet lamp and exposed to the radiations for 50 minutes. Alcohol was added occasionally to keep the solution in a liquid state. It was, however, allowed to dry up a few minutes before the end of the exposure. The dried residue after the exposure was made to 10 ml. with absolute alcohol and used as control. The solution was also shaken from time to time during the exposure. As during the dissolving of the residue the absolute alcohol comes in contact with atmospheric moisture, involving a source of error, it was compensated by exposing the non-irradiated portion of the solution of the unsaponifiable fraction to atmosphere and shaking, in a similar porcelain dish, for an equal interval of time (1 minute).

Spectrograms of both the solution containing vitamin A and the control were obtained simultaneously and the match point was observed with naked eye with the help of an illuminated disc.

Calculations (Zscheile and Henry, 1942) :—

$$E_{1 \text{ cm.}}^{1 \%} = \log \frac{I^{\circ}}{I} \times \frac{1}{cd},$$

where I° = the intensity of radiant energy transmitted through solvent filled cell,

I = intensity of the radiant energy transmitted by solution filled cell,

c = concentration in g. per 100 ml.

d = thickness of the solution layer in centimetres.

Conversion factors :—

$$E_{1 \text{ cm.}}^{1 \%} \times 1,600 = \text{International Units vitamin A.}$$

$$E_{1 \text{ cm.}}^{1 \%} \times 2,140 = \text{U.S.P. Units vitamin A.}$$

Apparatus used :—

Hilger quartz medium spectrograph E. 4 302 with the spectrum range from 2,100 to 7,000 Å° was used. The source of light was a hydrogen discharge tube built in the laboratory, and for destroying vitamin A in the control solution a Hanovia mercury vapour lamp operated

at 220 volts D.C. equivalent to 450 watts was used. The photographic plates used were Ilford process plates. These plates are slow and require an exposure of about 1 minute.

(A) *Vitamin A content of buffalo ghee.*

The majority of the samples examined in this investigation were prepared in the laboratory from the pure milk of various animals. Some samples were also obtained from houses and the local market. Of the market samples only those samples were assayed which appeared to be genuine ghee as shown by the common tests. For example they gave fairly high Reichert-Meissl and Reichert-Wolney values, and the refractive index was comparable to that of genuine buffalo ghee.

Method of preparation of ghee.—In India butter is isolated from the curd. The milk obtained from the animal in the morning is heated for two to three hours on a slow smoking fire and is allowed to boil once or twice for a short time. The milk obtained in the evening is just boiled and mixed with the morning milk in an earthenware vessel. A small quantity of diluted curd is churned with water and the butter is skimmed off from the top of the diluted curd. To clarify the butter into ghee, it is heated on a slow fire without stirring for about half an hour. Scum which gathers at the top is skimmed off. The clear fat so obtained is ghee.

The results of 48 samples assayed are shown in Table I :—

TABLE I.
Vitamin A content of different samples of buffalo ghee.

Description of the sample.	Number.	E $\frac{1}{1}$ % 1 cm.	U.S.P. Units per g.	I.U. per g.
I. <i>Genuine samples of ghee prepared in the laboratory from pure milk obtained from different animals.</i>	1	0.0180	38.5	30.40
	2	0.0150	32.0	24.00
	3	0.0145	31.0	23.20
	4	0.0130	28.0	20.80
	5	0.0101	22.0	16.16
	6	0.0101	22.0	16.16
	7	0.0105	22.5	16.8
	8	0.0091	19.0	14.56
	9	0.0143	31.0	22.88
	10	0.0143	31.0	22.88
	11	0.0128	28.0	20.48
	12	0.0125	27.0	20.00
	13	0.0151	32.0	24.16
	14	0.0152	32.0	24.32
	15	0.0155	33.0	24.80
	16	0.0160	34.0	25.60
	17	0.0188	40.0	30.08
	18	0.0204	44.0	32.64

TABLE I—*conold.*

Description of the sample.	Number.	E 1 % 1 cm.	U.S.P. Units per g.	I.U. per g.
I. <i>Genuine samples of ghee prepared in the laboratory from pure milk obtained from different animals.—conold.</i>	19	0·0172	37·0	27·52
	20	0·0188	40·0	30·08
	21	0·0202	43·0	32·32
	22	0·0204	44·0	32·64
	23	0·0220	47·0	35·20
	24	0·0233	50·0	37·28
	25	0·0165	35·0	26·40
	26	0·0134	29·0	21·44
	27	0·0148	32·0	23·68
	28	0·0161	34·0	25·76
	29	0·0166	35·0	26·56
	30	0·0155	33·0	24·80
	31	0·0166	35·0	26·56
	32	0·0153	32·8	24·48
	33	0·0157	34·0	25·12
	34	0·0160	34·0	25·60
	35	0·0155	33·0	24·80
	36	0·0143	31·0	22·88
	37	0·0146	35·0	23·36
	38	0·0236	50·0	37·76
II. <i>Genuine samples of ghee obtained from homes of people keeping buffaloes.</i>	39	0·0250	53·0	40·00
	40	0·0220	47·5	35·20
	41	0·0215	46·0	34·40
III. <i>Market samples of ghee—</i>				
(i) From Lahore market ...	42	0·0141	30·0	22·56
(ii) From Garh Shanker market	43	0·0126	27·0	20·16
(iii) From Sialkot market ...	44	0·0104	23·0	16·64
(iv) From Pindi Bhatian market	45	0·0133	28·0	21·28
(v) From Tandlianwala market	46	0·0121	26·0	19·36
(vi) From Gujranwala market ...	47	0·0131	28·0	20·96
(vii) From Gujranwala market ...	48	0·0154	33·0	24·64
AVERAGE ...			34·01	25·40

The vitamin A content of the samples prepared in the laboratory varied generally from 15 to 37 I.U. per g., the majority showing between 20 to 30 I.U. The samples of genuine buffalo ghee obtained from homes were found to have higher vitamin A content 34 to 40 I.U. per g. This may be due to the fact that animals in the homes are better looked after, and are well fed. The market samples were consistently lower in vitamin A content 16 to 24 I.U. per g. In the case of 4 samples very low values 14 to 16 I.U. were obtained. These were from animals which were towards the end of their lactation period and were weak and under-nourished.

The average value of the vitamin A content of buffalo ghee is found to be about 25.4 I.U. per g. In Table II the distribution of the samples according to their vitamin A content is shown :—

TABLE II.

Distribution of samples into different levels of vitamin A content.

	Samples prepared in the laboratory.	Samples from homes.	Samples from market.
Total number of samples	37	4	7
Number of samples of low vitamin content (below 24 I.U. per g.).	14	0	6
Number of samples of average vitamin content (24 to 30 I.U. per g.).	15	0	1
Number of samples of high vitamin content (above 30 I.U. per g.).	8	4	0

(B) *The effect of the method of preparation of ghee on vitamin A content.*

A sample of butter was divided into several portions and each portion was clarified by heating for varying lengths of time and at varying temperatures. The clear fat obtained at the end of the experiment was decanted off and its vitamin A content determined spectrographically. The results are shown in Tables III and IV :—

TABLE III.

Effect of method of preparation on the vitamin A content of ghee.

Sample of butter.	Vitamin A content of ghee I.U. per g. prepared from butter by heating at			
	150°C. to 200°C. for 3 min.	150°C. to 200°C. for 10 min.	60°C. for 25 min.	90°C. for 25 min.
1	22.88	22.88	20.00	20.48
2	24.32	24.32	25.60	24.80
3	30.08	32.64	30.08	27.52
4	32.32	32.64	37.76	35.00
5	25.60	26.40	26.40	24.80
6	...	26.40	24.32	21.44
7	24.80	25.60	24.80	25.60
8	22.88	26.40

TABLE IV.

Effect of method of preparation on the vitamin A content of ghee.

Sample of butter.	Time of heating, hours.	Temperature, °C.	Vitamin A content, I.U. per g.
1	2	60	22.88
	2	90	20.48
2	2	60	16.80
	2	90	16.80
3	2	60	16.80
	2	90	16.56
4	2	90	30.50
	4	60	20.00
5	2	90	24.32
	4	60	16.64

Table III does not indicate any significant difference in the vitamin A content of ghee prepared by exposure to temperature of 60°C., 90°C. or 150°C. to 200°C. for short periods of 10 to 25 minutes. The small differences observed are within experimental error. This shows that even relatively high temperatures do not cause any appreciable loss of vitamin A in ghee during the process of clarification. This is further brought out by the results summarized in Table IV. It will be seen that there is no significant difference in the vitamin A content of ghee samples prepared at 60°C. or 90°C. when the time of exposure is the same. A longer exposure of four hours, however, causes an appreciable loss even at a lower temperature. Samples of ghee prepared at 90°C. in two hours have about 30 per cent more vitamin A than those prepared at 60°C. in four hours from the same samples of butter. The loss of vitamin A is evidently due to slow atmospheric oxidation.

(C) The effect of storage on vitamin A in ghee.

Generally ghee is consumed within a month or two of its preparation. In the houses it is stored in tins and metallic or earthenware vessels and generally little attention is paid to the conditions of storage. From time to time, however, the ghee is examined for any smell arising from rancidity. If it develops rancidity it is re-melted and clarified again. The rancidity is evidently caused by moisture or any curds having been left behind in the ghee during clarification.

In this investigation in order to study the effect of storage the ghee was transferred to loosely stoppered glass-bottles which were stored in a cupboard in a room in the laboratory. Vitamin A content was estimated from time to time. The investigation covers about six months including the entire summer of a year when the temperature is high, and therefore maximum deterioration could have taken place. The results are shown in Table V.

TABLE V.

The effect of storage on the vitamin A content of ghee.

Sample of ghee.	Initial vitamin A content, I.U. per g.	Vitamin A content after the period of storage, I.U. per g.	Duration of storage, days.	Percentage loss.
1	10.64	10.64	3	0
2	13.40	13.40	3	0
3	22.56	24.32	10	0
4	30.40	30.60	15	0
5	20.00	19.40	26	3.7
6	32.64	30.60	119	11.4
7	31.50	30.08	119	7.0
8	30.08	30.06	122	2.5
9	32.64	26.96	122	18.2
10	35.20	26.40	122	25.2
11	37.76	30.60	122	22.0
12	22.88	16.64	130	25.8
13	24.32	16.64	172	28.1
14	30.40	20.00	175	29.8

Table V shows that there is no significant loss of vitamin A in ghee on storage for about a month. At the end of four months loss of vitamin A in different samples varied from 2.5 to 22.5 per cent. After five to six months the loss was nearly 25 to 30 per cent. Unfortunately, no observations could be made at the end of second and third months on account of vacation at the University.

Evidently incomplete clarification, the presence of moisture and the development of rancidity play a part in the deterioration of vitamin A content. If the ghee is clarified completely, has no traces of moisture and is stored in a manner so as not to expose it too much to the atmosphere, the destruction of vitamin A is much less. Those samples which developed traces of rancidity were found to have deteriorated comparatively to a greater degree.

These experiments indicate that under ordinary conditions of the storage of ghee in the homes there may be practically no loss in its vitamin A content at the end of one month. After two to three months the loss may be of the order of 5 to 10 per cent. After three to four months about 10 to 20 per cent and after five to six months as much as 30 per cent.

(D) *The effect of cooking on the vitamin A content of ghee.*

This problem has been studied by some authors. De and Majumdar (1938) using a spectrophotometric method assayed both vitamin A and provitamin A after cooking in water at a neutral pH, in an acidic medium by the addition of tamarind and in an alkaline medium by the addition of sodium bicarbonate. These conditions are somewhat different to the actual cooking conditions to which the fat is subjected before it is consumed in an average home.

In this investigation some common dishes were actually prepared with a slight excess of ghee. Ghee was then separated from the cooked food and assayed for its vitamin content. The following points are worth noting in this connection :—

- (i) Foodstuffs which contain high amounts of fat, e.g. meat, could not be used without separating their original fat.
- (ii) Turmeric and red chillies which impart a very strong colour to the ghee, making spectrographic assay of vitamin A impossible, had to be avoided in the cooking of the food.
- (iii) Even the natural pigments of the vegetables sometimes coloured the ghee intensively, particularly after their concentration in the unsaponifiable fraction. These solutions had a very strong general absorption in the ultra-violet region and the spectrographic assay could not be made. The antimony trichloride reaction was attempted in such cases with somewhat better success.

The results are shown in Table VI.

Many Indian methods of cooking involve frying in ghee. Frying is done either at the beginning or towards the end of cooking. The results summarized in Table VI show that frying causes a very considerable loss in vitamin A content. In the frying of *puree* 63 to 69.5 per cent vitamin A is lost. In the making of vegetable or dhal curries which also involve frying almost the whole of vitamin A is lost (experiments 4, 5 and 7). When frying is not done as in experiment 6, the loss is only about 20 per cent. It may be pointed out here that where almost total loss of vitamin A occurred, the time of cooking was also longer, 22 to 45 minutes, in addition to the inclusion of frying in the process of cooking.

In experiments 4, 5, 6 and 7 the fat was too highly coloured and spectrographic assay could not be undertaken. Only the antimony trichloride test was resorted to and it was found to be negative in the case of experiments 4, 5 and 7, while in the case of experiment 6 it showed a value which was about 80 per cent of the original.

In the making of *paranthas* which are made by the mixing of ghee to the dough and baking of the dough on a hot-plate like *chapattis*, the loss of vitamin A was only 8.7 per cent. Possibly the fact that the ghee was inside the dough and not exposed to the atmosphere protects its vitamin A content. In the case of *halwa* also which involves only roasting of semolina in ghee the loss was much less than frying being only 32 to 39 per cent. In the preparation of *pulao* the loss of vitamin A is also found to be 22 to 24 per cent.

These experiments also led to the conclusion that Indian sweets, practically all of which involve frying for long periods, would be completely devoid of vitamin A.

DISCUSSION.

As pointed out earlier, low values for the vitamin A content of butter and ghee of Indian cows have been reported in the literature. De (*loc. cit.*) and De and Majumdar (*loc. cit.*) obtained values as low as 14.1 I.U. per g. for butter-fat and 10.4 I.U. per g. for ghee. Sen

TABLE VI.

The effect of cooking upon the vitamin A content of ghee.

Number.	Name of food preparation.	Method of cooking.	Time taken in cooking, minutes.	Initial vitamin A content, I.U. per g.	Vitamin A content after cooking, I.U. per g.	Loss in vitamin A, per cent.	Remarks.
1	Fried purees	(a) Ghee heated nearly to boiling temperature and purees fried in it.	8	34.4	12.56	63.0	By spectrographic assay.
			6	34.4	12.56	63.0	
		(b) Further lot of purees fried in the ghee used in last experiment 1(a).	12	34.4	11.28	67.4	
			11	34.4	10.4	69.5	
2	Paranthas	Ghee is added to the dough which is baked on a hot-plate like chapattis.	5	34.4	31.84	8.7	By spectrographic assay.
			6	34.4	31.84	8.7	
3	Hahua	Semolina is roasted in ghee and then added to a syrup of sugar and cooked.	16	34.4	20.48	39.1	
			14	34.4	23.20	32.6	
4	Potato curry	Sliced potatoes are fried in ghee and then cooked in water with spices.	23	34.4	0	100.0	Negative antimony trichloride test.
			22	34.4	0	100.0	
5	Cauliflower curry	Pieces of cauliflower fried in ghee and then cooked in water containing spices.	24	34.4	0	100.0	
			30	34.4	0	100.0	
6	Sag curry	The leafy vegetable is cooked in water containing spices and then ghee added towards the end.	9	34.4	26.96	21.7	By antimony trichloride test.
			10	34.4	27.52	19.5	
7	Pulao curry	The pulao and the spices were fried in ghee and then cooked in water.	45	34.4	0	100.0	Negative antimony trichloride test.
			45	34.4	0	100.0	
8	Pulao	Onions and spices are fried in ghee to which water is added, brought to boil, and then rice added and cooked.	24	34.4	20.96	21.7	By spectrographic assay.
			21	34.4	20.4	23.9	

and Rai Sarkar (*loc. cit.*) could raise the vitamin A content of butter-fat only to 24 I.U. per g. after feeding their animals on carotene-rich fodders. Similar results have been reported by Ray *et al.* (*loc. cit.*). All these results referred to products obtained from the cow. The average values reported in the literature for the vitamin A content of butter-fat in Western countries is 50.6 ± 1.8 I.U. per g. (Sherman, 1937). Figures for cow's butter given by Boas Fixen and Roscoe (1937-38) who summarized work of many workers range from 10 to 38 I.U. per g. Our average value for the vitamin A content of buffalo ghee is found to be 25.4 I.U. per g.

The causes of these low values may be ascribed to several factors such as the breed of the animals, their state of nutrition and feeding, and the process of preparation of ghee. The process of preparation of ghee from milk involves heating at two stages. Milk from the morning milking is heated on a slow smoking fire for perhaps three to four hours. The second heating is at the time of the conversion of butter into ghee. Many earlier workers in this field had considered appreciable loss of vitamin A to be possible at this stage. This investigation has shown that during clarification of butter as ordinarily done in homes no appreciable loss of vitamin A is likely to occur. The effect of the first heating has not been studied in this investigation. It is likely that some loss may occur at that stage since it involves exposure of hot milk to the atmosphere for a long period of time. The low values obtained by earlier workers for buffalo ghee may be due to this loss. The samples studied by us were obtained from animals which were well fed, and this may be another reason for our obtaining higher values.

It has already been mentioned that somewhat low values of 14 to 16 I.U. per g. were obtained in the case of under-nourished animals towards the end of their lactation period. Lower values for the market samples may be due to unsatisfactory conditions of storage in tins, frequent remelting and also possible adulteration.

SUMMARY.

1. Forty-eight samples of buffalo ghee have been studied for their vitamin A content by the spectrographic method. Out of these, 41 samples were of genuine ghee prepared in the laboratory or in the homes from buffalo milk, while 7 samples were from the market and presumably pure. Twenty samples were found to contain below 24 I.U. of vitamin A per g.; 16 samples showed values between 24 and 30 I.U. and 12 samples above 30 I.U. per g. The average of all the samples taken together was found to be 25.4 I.U. per g.

2. Tests in the laboratory indicated that the process of clarification of butter into ghee ordinarily used in Indian homes is not likely to cause any loss of vitamin A. Prolonged heating at a relatively low temperature causes greater destruction of vitamin A than heating to high temperatures for short intervals.

3. Storing of samples of ghee under ordinary conditions at room temperatures in the summer in Lahore did not result in any significant loss of vitamin A activity during the course of a month. At the end of four months loss varying from 2.5 to 22.5 per cent was noticed in the different samples and after five to six months the loss was between 25 and 30 per cent.

4. The effect of different Indian methods of cooking upon the vitamin A content of ghee was also investigated. In the frying of *purees* 63 to 69.5 per cent of vitamin A was lost. In the making of vegetables and dhal curries, which involve the frying of vegetables and spices in ghee for 22 to 45 minutes, there appeared to be a total loss of vitamin A, while in ordinary cooking in the presence of water the loss was only about 20 to 24 per cent. In *paranthas* the loss was only 8.7 per cent and in *halwa* as much as 32 to 39 per cent.

The authors desire to express their gratitude to Dr. W. R. Aykroyd for suggesting this study and to Dr. A. H. Butt, Director of Public Health, Punjab, for providing all the funds required for this investigation.

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THE EFFECT OF HESPERIDIN AND A FACTOR IN BENGAL GRAM (*CICER ARIETINUM*) ON THE GROWTH OF GUINEA-PIGS.

BY

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(From the Nutrition Research Laboratories, I. R. F. A., Coonoor, S. India.)

[Received for publication, August 1, 1945.]

In a previous paper (Kamala Bhagvat and Rao, 1942) it was shown that dry unsprouted Bengal gram contained, in addition to vitamin C, an alkali-stable growth factor for guinea-pigs. The guinea-pigs with scurvy recovered when fed dry Bengal gram. The capillary resistance of these animals was measured according to the method described by Bacharach, Coates and Middleton (1942) and was found to increase more rapidly in animals receiving Bengal gram than in others receiving pure vitamin C alone. This suggested that Bengal gram might contain a factor identical with vitamin P or hesperidin. Armentano, Bentsath, Beres, Rusznyak and Szent-Györgyi (1936), Zacho (1939), Scarborough (1939) and Rusznyak and Benko (1941) have reported that hesperidin increases capillary resistance in guinea-pigs and in men. There is, however, little information about the effect of hesperidin on the growth of guinea-pigs. In the present investigation the effect of hesperidin on the growth of guinea-pigs receiving a diet containing sufficiency of vitamin C has been investigated. An attempt has been made to isolate the factor in Bengal gram which influences capillary resistance, and the growth-promoting effect of this factor and that of hesperidin have been compared.

EXPERIMENTAL.

Isolation of the factor in Bengal gram.—The method used was that employed by Szent-Györgyi (1938) for the isolation of citrin from lemons. It was carried out as follows: Dry unsprouted Bengal gram was finely powdered and the powder was extracted twice for 24 hours with 3 volumes of 96 per cent alcohol. The suspension was filtered and the filtrate was treated with barium and lead acetates in order to remove interfering substances. The precipitate was removed by filtration and rejected. The filtrate was then made alkaline by the addition of ammonium hydroxide. The precipitate thus formed was removed on the centrifuge and dissolved in 10 per cent acetic acid. This solution contained the active factor. It was purified by re-precipitation with ammonium hydroxide and was then suspended in 96 per cent alcohol to which dilute sulphuric acid was added. A precipitate of lead sulphate was formed which was removed on the centrifuge. The factor went into solution. One ml. of this solution, containing 12 mg. of total solids, was used directly for the feeding experiments, no attempts being made at further purification.

The pure re-crystallized hesperidin used in these experiments was obtained through the kindness of Dr. A. L. Bacharach from the Glaxo Laboratories, Ltd., Greenford, England.

Experiments were carried out on young guinea-pigs, weighing from 200 g. to 260 g. The animals were kept on a stock diet rich in vitamin C for a week, after which they were put on the experimental diet. The composition of the latter was as follows:—

Parts.			Parts.		
Crushed oats	...	31	Shark-liver oil	...	1
Atta	...	31	NaCl	...	1
Skimmed milk powder	...	30	Yeast	...	1
Gingelly oil	...	5			
TOTAL					100

The diet was moistened with water and given in the form of balls. The various experimental groups were as follows :—

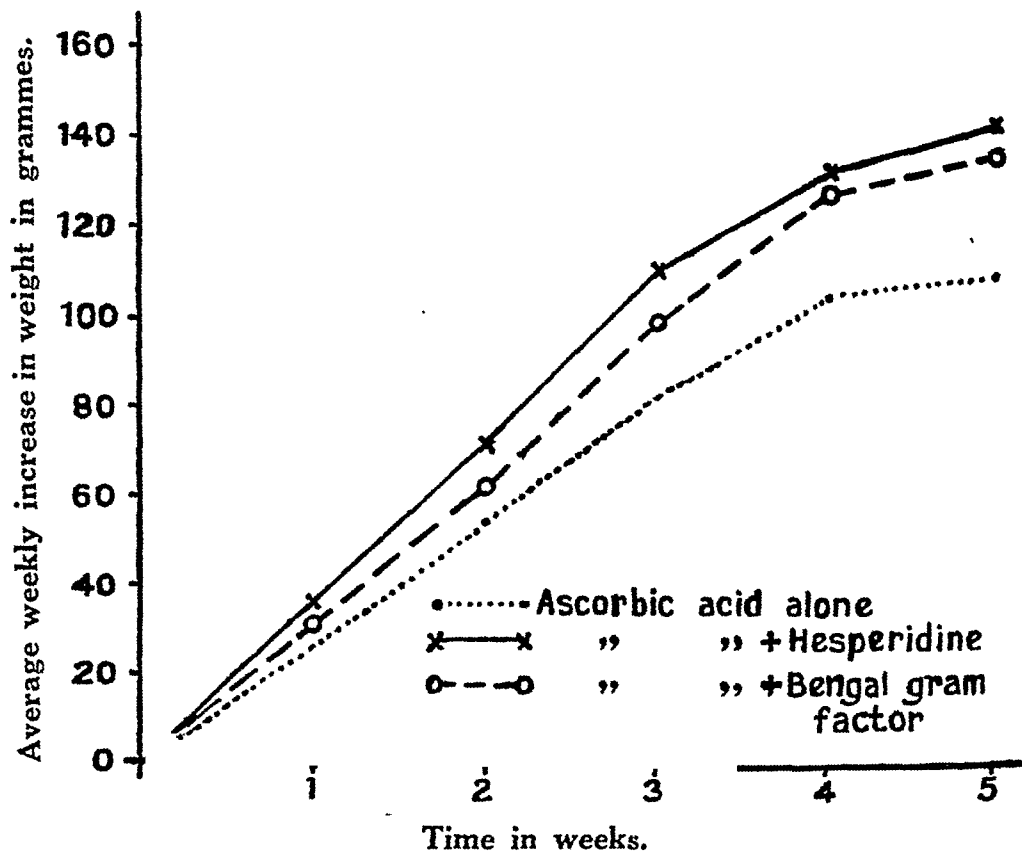
TABLE

Groups.				Number of animals.
I.	Negative controls—(animals receiving the basal diet alone)	6
II.	Positive controls—(receiving the basal diet + 5 mg. ascorbic acid per animal per day)	16
III.	Animals receiving the Bengal gram factor—(1 c.c. daily per animal)	6
IV.	Same as III + 5 mg. of ascorbic acid per animal per day	18
V.	Animals receiving 5 mg. hesperidin per animal per day	6
VI.	Same as V + 5 mg. of ascorbic acid per animal per day	18

Three different sets of experiments were carried out in which animals were fed on the various diets described above. The number of animals shown represents the total number included in the 3 series of experiments. Average weekly increase in weight in groups II, IV and VI is shown in the Graph. The animals in groups I, III and V, which received no vitamin C, died within 20 to 28 days, and post-mortem examination revealed typical signs of scurvy. Their weight curves are not shown in the Graph :—

GRAPH.

Effect of hesperidin and Bengal gram factor on the growth of guinea-pigs.



The guinea-pigs receiving hesperidin and vitamin C showed more rapid increase in weight than those receiving vitamin C alone, suggesting that hesperidin is a necessary factor

for the growth of this species. Those given the Bengal gram factor also showed increases in weight of a similar order to that of the group receiving hesperidin. The method followed in the purification of this factor and its chemical nature suggest that it is identical with hesperidin. Attempts are now being made to develop a biological method, based on the growth of guinea-pigs, for the assay of hesperidin in foods.

SUMMARY.

1. Hesperidin or vitamin P appears to be essential for the proper growth of guinea-pigs.
2. A factor isolated from Bengal gram (*Cicer arietinum*) was found to have a similar growth-promoting effect and may be identical with hesperidin.

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STUDIES ON THE DESTRUCTION OF VITAMIN A IN SHARK-LIVER OIL.

Part VI.

SOME FACTORS AFFECTING THE STABILITY.

BY

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[Received for publication, May 30, 1945.]

THE influence of moisture, free fatty acids and dilution with ground-nut oil on the development of rancidity and destruction of vitamin A in shark-liver oil has been studied. It has been established beyond doubt that heat, light and metals accelerate oxidative changes in fats and oils. Data regarding the rôle of moisture are, however, conflicting. Greenbank and Holm (1924) observed that water (about 4 per cent) in butter-fat and lard materially increased the induction period. Greenbank (1936) subsequently found that at a low concentration (0.15 per cent) water acted as a pro-oxidant. He observed further that the same substance may be either an anti-oxidant or a pro-oxidant depending upon its state of dissociation. Moisture which causes dissociation of the anti-oxidants may thus act indirectly. Holmes, Corbett and Hartzler (1936) reported that the time taken for the complete destruction of vitamin A in halibut-liver oil containing hydroquinone and lecithin at various levels was consistently increased in the presence of water (about 7 per cent). Newton (1932) on the contrary found that moisture destroyed the activity of anti-oxidants.

Literature on the influence of fatty acids on the development of rancidity in fats and oils is meagre and inconclusive. Rogers *et al.* (1909, 1912) observed that acidity had a decidedly adverse effect on the keeping quality of butter. Greenbank and Holm (*loc. cit.*) also reported that fatty acids catalysed the oxidation of butter, and that acids of higher molecular weight were more active than those of lower molecular weight. More recently, Lea (1938) failed to note any such catalytic effect, but these experiments were carried out in the presence of water and this might explain the discrepancy.

Dilution of shark-liver oil with ground-nut oil with a view to producing oils of uniform potency is being adopted by some producers in India. It was of interest, therefore, to study the influence of ground-nut oil on the stability of shark-liver oil. Recently, Holmes and Pigott (1942) observed that vitamin A was more stable in cod-liver oil than in cotton-seed, ground-nut or corn oils and suggested that cod-liver oil may contain some natural anti-oxidants, or that its vitamin A may be in a more stable form than that of the vitamin A concentrate incorporated in the vegetable oils.

EXPERIMENTAL.

Shark-liver oil was dried by filtering through a column of anhydrous sodium sulphate (1.3 cm. diameter and 5 cm. height for 30 c.c. of oil). During the filtration, the oil was maintained at 18°C. for effective dehydration. The moisture and volatile matter, estimated by the loss in weight of 5 c.c. of oil maintained at 75°C. and 25 mm. pressure for one hour, were usually less than 0.1 per cent. A portion of the dried oil was shaken up with a few drops of water and the water separated by centrifuging. Thus, a 'moist' oil containing about 0.2 to 0.25 per cent of moisture was obtained. To another portion of the dried oil water (1 or 2 per cent of the oil by volume) was added. Ten c.c. of each of these—dry, moist and with water—were taken in specimen tubes (2.2 cm. diameter and 7.5 cm. height) to which trap-tubes containing anhydrous calcium chloride were attached to prevent the absorption of water by the oil from the atmosphere. They were maintained in an incubator at 40°C.

and after a definite interval the oils were analysed for peroxide number, acid value and vitamin A content (Dattatreya Rao, 1944, 1945, 1945a). The experiment was repeated with six other samples of oil and the results are given in Table I. With samples S, B and L, only 5 c.c. of oil were used, the other experimental conditions being the same, so as to produce appreciable oxidative changes in lesser time.

TABLE I.

Influence of moisture on the oxidative changes in shark-liver oil.

Sample of oil.			Moisture, per cent.	Period of storage, days.	Peroxide number.	Acid value.	Vitamin A, I.U. per g.
F dry	0.096	0	0	5.46	4,360
F dry	0.096	15	19.3	5.81	4,100
F moist	0.224	15	18.5	5.84	4,050
F + water	Over 2	15	17.5	5.85	4,150
XII dry	0.071	0	0.5	0.6	11,700
XII dry	0.071	17	20.1	1.51	10,370
XII moist	0.243	17	20.5	...	10,470
XII + water	Over 1	17	19.5	0.83	10,470
XI dry	0	1.0	2.76	6,890
XI dry	27	20.3	4.28	...
XI moist	27	19.8
XI + water	Over 1	27	20.3	3.34	...
XV dry	0	0.5	0.15	690
XV dry	24	17.5	6.07	410
XV moist	24	17.1	...	390
XV + water	Over 2	24	16.8	5.27	400
S dry	0.083	0	0.3	0.19	28,520
S dry	0.083	10	12.0	0.96	23,680
S moist	0.215	10	10.8	0.77	22,400
S + water	Over 2	10	11.5	0.94	22,700
B dry	0.102	0	1.5	0.45	4,700
B dry	0.102	12	28.0	1.73	4,030
B moist	0.247	12	25.8	1.54	4,140
B + water	Over 2	12	27.3	1.48	4,190

TABLE I—*concl'd.*

Sample of oil.	Moisture, per cent.	Period of storage, days.	Peroxide number.	Acid value.	Vitamin A, I.U. per g.
L dry	0	0.5	0.34	610
L dry	10	24.3	3.25	335
L moist	10	24.5	2.86	335
L + water ...	Over 1	10	23.3	2.77	350

It will be observed that the acid value of samples F and XI is rather high. They were included in the experiments since they typify the condition of some oils that are being produced in India at the present time. As pointed out in a previous part, the high acidity results from enzyme action during the delay in the extraction of the oil after collecting the livers.

The increase in peroxide number and the amount of vitamin A destroyed in three samples of each batch after storage were practically the same indicating that water had little effect, if any, on the rate of oxidation.

The fatty acids from shark-liver oil were prepared by the following method: 1.5 g. to 2 g. were saponified by boiling for 15 minutes under reflux with 20 c.c. of 10 per cent alcoholic potash. The solution was diluted with 40 c.c. of water and extracted four times with freshly distilled ether to remove the unsaponifiable matter. The fifth extract, after washing with water and drying with anhydrous sodium sulphate, left no residue on evaporation. The alcoholic solution was then acidified with dilute sulphuric acid and the liberated fatty acids were extracted with ether. The ether solution was washed and dried, and evaporated in a current of nitrogen. The residue was dissolved in about 30 g. of the same sample of shark-liver oil. The acid value of the oil was determined by titration in hot alcohol against 0.1N alkali using phenolphthalein as the indicator. Accelerated oxidation tests were carried out at 40°C. as described before (Dattatreya Rao, 1945, 1945a) with these samples of oil—original and acidified—and the induction period determined from the curves for the development of peroxides. The inflexions in the curves generally occurred near peroxide number 25 and hence the time required to reach this peroxide number has been taken as the induction period. The experiment was carried out with two other samples of oil also and the results are presented in Tables II and III:—

TABLE II.

Influence of fatty acids on peroxide development and vitamin A destruction.

Period of storage, hours.	Peroxide number.		Percentage of vitamin A destroyed.	
	Oil U	Oil U + acids	Oil U	Oil U + acids
0	0	0
72	14.0	15.3	2	6
144	22.0	24.5	15.3	16.7
216	38.8	47.3	30.6	38.8
264	73.5	83.8	50.2	55.0

TABLE II—*concl'd.*

Period of storage, hours.	Peroxide number.		Percentage of vitamin A destroyed.	
	Oil IV	Oil IV + acids	Oil IV	Oil IV + acids
0	0	0
72	19.5	20.8
120	25.5	28.5	17.4	24.0
168	38.5	45.5	32.0	38.0
240	65.8	85.3	47	57.0
316	95.3	115.0

	Oil D	Oil D + acids	Oil D	Oil D + acids
0	0	0
72	13.5	15.0	12.0	24.0
144	27.0	31.8	20.5	24.0
192	55.3	72.5	37.0	43.5
240	106.0	124.0	60.0	59.0

TABLE III.

Influence of fatty acids on the stability of shark-liver oil.

Sample of oil.		Acid value.	Induction period, hours.
U	1.32
U + acids	8.41
IV	0.13
IV + acids	10.5
D	0.62
D + acids	9.16

It may be observed that the induction period of the samples containing the added fatty acids was consistently shorter than that of the original oil, showing that fatty acids act as mild catalysts in the oxidation of vitamin A and the glycerides in shark-liver oil.

Three samples of neutralized and bleached ground-nut oil were mixed with three samples of fresh shark-liver oil in different combinations. The rates of formation of peroxides in the original and mixed samples of oils when exposed in thin layers at 40°C. to atmospheric

oxidation were determined as usual. The periods of induction, i.e. the time taken to reach a peroxide number of 25, were measured from the time-peroxide number curves. The results are summarized in Table IV :--

TABLE IV.

Effect of ground-nut oil on the development of rancidity in shark-liver oil.

Oil.				Vitamin A, I.U. per g.	Induction period, hours.
Ground-nut oil (G.O.)	I	0	528
"	"	II	...	0	430
"	"	III	...	0	116
Shark-liver oil (S.L.O.)	A	12,000	134
"	"	E	...	21,800	124
"	"	F	...	5,000	132
G.O. I (3 parts) + S.L.O. A (1 part)			328
"	"	+ S.L.O. E (1 part)	290
"	"	+ S.L.O. F (1 part)	304
G.O. II (3 parts) + S.L.O. A (1 part)			296
"	"	+ S.L.O. E (1 part)	274
"	"	+ S.L.O. F (1 part)	288
G.O. III (2 parts) + S.L.O. A (1 part)			126

Ground-nut oils I and II were freshly refined, but ground-nut oil III was stored after refining under factory conditions for some months during which it must have gone rancid as shown by the low induction period. The latter actually increased the rate of oxidation of shark-liver oil while the former stabilized it considerably.

DISCUSSION.

Water had practically no effect on oxidative deterioration in shark-liver oil (Table I). Mild anti-oxidants of the acid and amine type (Lea, 1936 ; Olcott and Mattill, 1936) which have pronounced action in vegetable oils lose their efficiency as the unsaturation of the fats increase and have little effect on fish oils (Brocklesby, 1941). Similarly, the slight anti-oxidative action of water observed with lard and butter may not be appreciable in the highly unsaturated shark-liver oil.

The results in Table I raise another interesting point : Greenbank and Holm (*loc. cit.*) found that, in the presence of moisture, compounds which are responsible for the tallowy odour (aldehydes and ketones) in rancidity show a tendency to decrease. They postulated that in the presence of moisture the oxidation proceeded to the acid stage, but in its absence the products formed were aldehydes and ketones. The experiments with shark-liver oil, however, indicate that the increase in free fatty acidity is greater in the dry than in the moist condition during the development of rancidity.

Free fatty acids in shark-liver oil hasten the development of rancidity and the destruction of vitamin A (Tables II and III). The results are in confirmation of the observations of Greenbank and Holm (*loc. cit.*) with butter-fat and lard. It was shown before (Dattatreya

Rao and Banerjee, 1944) that storage of livers after removal from the sharks increased the acid value of the resulting oil. Obviously, therefore, oil prepared from preserved livers may be expected to be less stable than oil from fresh livers.

Bulk samples of shark-liver oil generally have an average potency of 5,000 I.U. to 6,000 I.U. per gramme, and to bring down this value to the level indicated by Ranganathan (1941) nearly three to four times its weight of ground-nut oil has to be used for dilution. Added in these proportions, freshly refined ground-nut oil retarded the deterioration of shark-liver oil (Table IV). This effect is to be expected since ground-nut oil has a much greater induction period and is less susceptible to oxidative rancidity than shark-liver oil. Vegetable oils, in general, are more stable than fish-liver oils because they are less unsaturated and also contain larger amounts of natural anti-oxidants (Brocklesby, *loc. cit.*).

In spite of the beneficial effect on the stability, the author considers the 'adulteration' undesirable. The price per unit of vitamin A increases as the cost of the unwanted ground-nut oil also has to be met. Further, this practice gives scope for unscrupulous exploitation. In this laboratory we have often examined samples of commercial shark-liver oil containing hardly 100 I.U. of vitamin A. No doubt, vitamin A potency of shark-liver oil varies considerably; but it should be possible, with little difficulty, to blend different samples judiciously to get a fairly uniform product for the market.

SUMMARY.

The influence of moisture, free fatty acids and ground-nut oil on the development of rancidity and destruction of vitamin A in shark-liver oil have been studied using an accelerated oxidation method. Water was practically inert and the increase in free fatty acids during oxidative rancidity was greater in the presence of moisture than in the dry oil. Free fatty acids acted as mild catalysts in the oxidation of the glycerides and the vitamin. The induction period of shark-liver oil, as determined by the formation of peroxides, was increased when the oil was mixed with freshly refined ground-nut oil.

The author has great pleasure in thanking Mr. B. N. Banerjee and Professor V. Subrahmanyam for their helpful suggestions and keen interest throughout these investigations.

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FLUORINE AND DENTAL CARIES IN INDIA.

BY

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THE correlation between the incidence in children of dental caries, mottled enamel and the fluorine content of drinking water has been studied by a number of workers (Dean, 1938, 1940; Dean, Jay, Arnold, Jr., McClure and Elvove, 1939; Dean, Jay, Arnold, Jr., and Elvove, 1941; Dean, Arnold, Jr., and Elvove, 1942; McClure, 1941; Ockerse, 1941; Sognnaes and Armstrong, 1941; Wilson, 1941; McClendon, Foster and Supplee, 1942; Murray and Wilson, 1942). The same problem has been studied in adults by Deatherage (1943). In India it has been investigated by Shortt, Barnard and Nayar (1939) and by Day (1940). All these workers support the view that there is an inverse correlation between the incidence of dental caries and the fluorine concentration of drinking water. Since dental caries is widely prevalent in certain parts of the world, the reported lower incidence resulting from a high intake of fluorine associated with mottled enamel has attracted much interest, and suggestions have been made that dental caries could be controlled by means of the regular ingestion of this element. In India the incidence of dental caries is much lower than in Northern Europe and America and fluorosis is endemic in certain areas. It was felt to be of considerable interest and importance to study the relation between dental caries and fluorosis in India. In the present investigation the following problems have been studied:—

- (a) The relation, if any, between the degree and severity of mottling and the concentration of fluoride in drinking water.
- (b) The incidence of dental caries in groups (both children and adults) with and without mottled enamel.
- (c) The incidence of dental caries in the presence of various degrees of severity of mottling.

Method of recording dental caries and mottled enamel.

This was the same as that followed in earlier surveys (Shourie, 1941, 1942). A detailed dental inspection, in which all the available tooth surfaces in the mouth were examined using a dental mirror and probe, was made in the case of each individual. The total number of the teeth and the number of deciduous and permanent teeth (when present) were recorded. All extracted teeth were recorded as carious, except when known to have been lost as the result of trauma or extracted for some other reason. In recording the extent of caries the classification of Day and Sedwick (1934) was employed, which is as follows:—

1. Initial caries including softened or discoloured pits and fissures giving lodgment to fine explorers.
2. Freely accessible approximate cavities and small open cavities involving less than one-quarter of the tooth.
3. More extensive caries involving more than one-fourth and less than two-thirds of the crown.
4. Caries involving from two-thirds to complete destruction of the crown.

The average caries figure = $\frac{\text{Total caries figures}}{\text{Number of teeth examined}}$

The total caries figure is calculated by multiplying the number of teeth under each of the heads mentioned above by the corresponding number, i.e. 1. 2. 3 or 4, as the case may be.

The degree of severity of mottled enamel was recorded according to the classification of Dean, Dixon and Cohen (1935): 1. Normal. 2. Questionable. 3. Very mild. 4. Mild. 5. Moderate. 6. Moderately severe. 7. Severe.

GROUPS STUDIED.

Group 1.—Children were examined in rural areas within a radius of 50 miles from Lahore City (Punjab). Fluorosis is endemic in this part of the Punjab. The number of children examined was 1,223, attending schools in 15 villages. Age ranged from 6 to 15. The mode of life, customs and dietary habits of all the children were similar. Only children who had lived in the area since birth or a few months after birth were included in the investigation. Schools in rural areas do not close for vacations for more than 3 to 4 weeks at a time so that the children of school-going age rarely leave their villages.

Samples of water habitually drunk by children were collected—129 samples in all—and the fluoride content estimated according to Sanchis' (1934) method. In rural areas there is no communal water-supply and water for drinking or domestic use is obtained from shallow wells or shallow tube-wells worked by hand.

Group 2.—In the Kurnool district of the Madras Presidency, where fluorosis is prevalent, the incidence of dental caries in 831 children showing mottled enamel was determined. This was compared with the incidence of dental caries in 4,044 children in other parts of Madras and in Central India showing no mottled enamel.

Group 3.—A group of 1,074 healthy members of the Punjab Police Force were examined for mottling and dental decay and the incidence of caries in those with or without mottling was compared.

RESULTS.

1. *Fluorine and mottling.*—(a) Table I shows the incidence of mottled enamel in 296 children of group 1 divided into 19 sub-groups. The members of each sub-group were drinking water of known fluorine content. Sub-groups in which the number of children drinking water from the same source was less than nine are not included in Table I :—

TABLE I.

Concentration of fluorine in drinking water and the corresponding degree of mottled enamel in children.

Serial number.	Sample number.	Fluorine in water (p.p.m.).	Total number of children examined.	DEGREE OF MOTTLING.							PERCENTAGE SHOWING MOTTLING.	
				Normal.	Questionable.	Very mild.	Mild.	Moderate.	Moderately severe.	Severe.	Children with questionable mottling included.	Children with questionable mottling excluded.
1	1	3.0	35	...	5	14	9	5	2	...	100	86
2	5	0.7	19	...	2	10	6	1	100	89
3	16	4.8	23	1	2	2	12	1	5	...	96	87
4	22	0.6	17	6	5	3	1	1	1	...	65	35
5	31	1.8	20	3	...	9	3	4	1	...	85	35
6	34	1.25	10	3	2	4	1	70	50
7	35	1.2	11	3	5	2	1	73	27
8	43	1.0	11	4	3	2	1	1	64	36
9	45	1.6	18	3	3	4	4	4	83	67
10	47	1.0	9	1	3	3	...	2	89	56
11	52	1.5	9	1	2	5	1	89	67
12	60	3.0	17	3	1	6	3	4	82	76
13	73	2.8	14	3	3	3	3	2	79	57
14	77	2.1	10	...	3	6	...	1	100	70
15	94	0.4	9	4	1	2	1	1	56	44
16	99	0.4	18	15	1	2	17	11
17	10	1.0	13	5	6	2	62	15
18	121	1.2	20	2	4	10	4	90	70
19	125	1.4	13	5	3	2	2	1	62	38

The coefficient of correlation between fluorine concentration and the percentage of children showing mottled enamel was calculated and found to be + 0.552 (nearly 1 per cent level of significance). Children showing 'questionable mottling' were excluded from the calculation. When the latter were included the correlation coefficient became + 0.608 (less than 1 per cent level of significance). Both these values are statistically significant and indicate a positive correlation between the incidence of mottled enamel and the fluorine content of drinking water.

(b) The question whether the severity of mottling increased with increasing concentration of fluorine was investigated. The figures in Table I, relating to the fluorine content of water, were divided into 2 groups, viz. 0.0 to 1.99 and 2.0 and above (p.p.m. respectively), and the severity of mottling associated with these concentrations was estimated. This pooling of data was considered advisable because of the small numbers in certain sub-groups which figure in Table I. The results are given in Table II:—

TABLE II.

Concentration of fluorine and severity of mottling in children.

Fluorine concentration in water (p.p.m.).	DEGREE OF MOTTLING.					TOTAL.
	Normal.	Questionable.	Very mild.	Mild.	Moderately severe or severe.	
From 0.0 to 1.9	55	40	60	25	17	197
Above 2.0	7	14	31	27	20	99
TOTAL	62	54	91	52	37	296

Statistical analysis indicated that the severer degrees of mottled enamel were associated with the higher concentration of fluorine. The value of χ^2 with only 4 degrees of freedom was 30.09 which is highly significant.

2. *Caries and mottled enamel in children.*—(a) The incidence of caries in group 1 (all children) is given in Table III which shows the number and percentage of deciduous and permanent teeth carious. In the total of 30,789 teeth examined, caries was present in 6.1 per cent. Day (*loc. cit.*) carried out the dental examination of 203 children in Kasur, a small town situated some 35 miles from Lahore in the area in which fluorosis is endemic and found that of the 5,237 teeth 8.67 per cent were carious. The population of Kasur may be regarded as semi-rural as compared with the purely rural groups studied in the present investigation. The percentage of rural children found entirely free from caries was 45.7 as compared with 41.8 observed by Day (*loc. cit.*) in Kasur. Bull (1943) reported that 29.8 per cent of 1,647 children in an endemic fluorosis area in U.S.A. were free from dental decay. The degree of fluorosis in these groups may not have been the same. It is, however, probable that among populations equally exposed to excess of fluorine, other factors, e.g. the nature of the diet, will influence the incidence of dental caries.

TABLE III.

Incidence of dental decay in children in an endemic fluorosis area in the Punjab.

Age.	Number of children examined.	Percentage of children free of caries.	TEETH CARIOUS.				
			DECIDUOUS.		PERMANENT.		ALL TEETH.
			Number.	Per cent.	Number.	Per cent.	Percentage carious.
6	71	51.4	1,203	5.4	468	2.8	4.8
7	158	43.6	2,390	11.5	1,411	2.7	8.3
8	206	44.1	2,773	9.6	2,230	3.1	6.6
9	198	41.4	2,244	10.2	2,629	2.8	6.2
10	187	43.8	1,660	11.3	2,998	3.3	6.1
11	138	44.9	778	14.3	2,772	3.6	6.0
12	93	51.6	412	11.1	2,024	5.1	6.0
13	89	56.1	181	8.9	2,242	3.0	3.4
14	44	45.4	54	12.9	1,267	4.6	4.9
15	18	44.4	11	...	477	4.4	4.3
16	21	57.1	565	6.0	6.0

	Total number of teeth.	Percentage carious.	Extent of caries.				Average caries figure.
			1	2	3	4	
Deciduous ...	11,706	10.3	418	395	190	205	0.22
Permanent ...	19,083	3.6	442	174	27	44	0.05
TOTAL ...	30,789	6.1	860	569	217	249	0.12

(b) In Table IV is recorded the incidence of dental decay in children (in the endemic fluorosis area) with and without mottled enamel. Children with mottling of enamel showed more freedom from caries in comparison with those showing no mottling in all age groups except age group 12.

TABLE IV.

Incidence of dental decay in children with and without mottled enamel in an endemic fluorosis area.

Age.			6	7	8	9	10	11	12	13	14	15	
Not mottled	{	Number of children	...	23	37	45	45	30	31	21	17	12	7
		Per cent free of caries	...	47.8	27.0	31.1	26.6	40.0	41.6	57.1	41.1	41.6	42.8
Mottled	{	Number of children	...	48	121	161	153	157	107	72	72	32	11
		Per cent free of caries	...	52.1	47.7	47.8	45.7	44.6	45.8	50.0	61.1	46.8	63.6

(c) In Table V the incidence of dental caries in children in South India with and without mottled enamel is compared. The group of children with mottled enamel were resident in the Kurnool area, while the remainder, free from mottling, were living in other parts of South India. A number living in Central India are also included. The groups are, therefore, not strictly comparable as regards diet and living conditions. Perusal of Table V shows that the incidence of dental caries in the different age groups was consistently higher in the children not showing mottled enamel.

TABLE V.

Dental caries in children with and without mottled enamel in South and Central India.

Serial number.	Age.	NUMBER OF CHILDREN.		PERCENTAGE OF CHILDREN FREE FROM CARIES.		Difference between columns (5) and (6).
		With mottling.	Without mottling.	With mottling.	Without mottling.	
(1)	(2)	(3)	(4)	(5)	(6)	(7)
1	5	5	37	40.0	43.2	- 3.2
2	6	23	184	52.2	51.1	+ 1.1
3	7	39	352	51.3	42.0	+ 9.3
4	8	115	396	42.6	36.8	+ 5.8
5	9	57	347	52.6	34.5	+18.1
6	10	107	378	49.5	42.3	+ 7.2
7	11	45	320	60.0	38.4	+21.6
8	12	121	377	46.3	33.9	+12.4
9	13	62	314	48.4	37.2	+11.2
10	14	76	292	47.4	35.6	+11.8
11	15	55	303	43.6	35.3	+ 8.3
12	16	59	277	49.1	31.0	+18.1
13	17	67	467	56.7	37.2	+19.5

Except in the case of age group 5, all the figures in column 7 bear a *plus* sign. The analysis of variance due to various factors will throw light on the statistical significance of these differences. The results of such analysis are shown below :—

Source of variance.				Degree of freedom.	Sum of squares.	Variance.
				(1)	(2)	(3)
Age	12	375.82	31.32
Mottling v. non-mottling		1	776.83	776.83
Residual	12	319.56	26.63
TOTAL				25	1,472.2	...

Column 3 shows the degree of variability due to the three causes. Residual variance, arising from uncontrolled factors, and variance due to age, were small in relation to variance due to mottling. The latter was many times greater than that attributable to the factor of chance. This clearly suggests that the presence of mottling is protective against dental caries. A further point of importance emerges from statistical study of the data. As age increases there is a tendency for the differences to increase in magnitude. The slope of a straight line fitted to these values was calculated by the method of least squares and its statistical significance tested. The value was found to be 1.245, with a standard error of 0.541. This is significant at a 5 per cent probability level, and indicates that the nature of the slope could not be due to chance. It seems justifiable to draw the conclusion that, within the age span in question, children with mottled enamel show an increasing advantage over those without mottled enamel as regards freedom from caries as age advances.

(d) *Caries and degree of mottled enamel.*—The relation between the incidence of dental caries and the degree of mottling in the different age groups in group 1 was also investigated and different statistical tests were employed. Although in the earlier analysis it was established that mottling is associated with a lower incidence of dental caries this examination failed to show that the severer forms of mottling were related to a comparatively lower incidence of caries. It is possible that fluorine may exert a protective effect against caries in concentrations below those necessary to produce the severe degrees of mottled enamel.

3. *Caries and mottled enamel in adults.*—Table VI shows the incidence of dental caries in 1,074 adults with and without mottled enamel. In this case no association between the incidence of dental caries and mottling was elicited. The difference in the incidence of caries in those with and without mottled enamel was statistically significant only in one age group, i.e. 31 to 40. The lack of association between dental caries and mottling may be due to the fact that the policemen in question had not been living throughout their life in an endemic fluorosis area. It is possible that with change in water-supply the fluorine present in dental and other tissues is lost by excretion, while the mottling which is produced by excess ingestion of fluorine during the early years of life remains as a permanent feature.

TABLE VI.

Incidence of dental caries in adults (Punjab Police) with and without mottled enamel.

(a)

Age group, years.	With or without mottling.	Number of adults examined.	Total number of teeth.	Percentage of adults free from caries.	Percentage of teeth carious.
18 to 20	With mottling ...	65	1,979	38.4	7.5
	Without mottling ...	106	4,560	29.8	8.9
21 to 30	With mottling ...	121	3,789	30.5	8.7
	Without mottling ...	401	12,548	30.6	10.4
31 to 40	With mottling ...	33	1,042	48.4	9.1
	Without mottling ...	177	5,559	26.5	10.8
41 to 50	With mottling ...	8	308	50.0	5.5
	Without mottling ...	92	2,903	29.3	8.9
51 to 55	With mottling ...	1	32	...	9.3
	Without mottling ...	7	224	42.8	9.3

(b)

		Total number of teeth.	Percentage of carious teeth.	Extent of caries.				Average caries figure.	Number of carious teeth per mouth.
				1	2	3	4		
All ages	With mottling ...	7,150	8.3	375	92	30	103	0.15	2.6
	Without mottling ...	25,794	10.1	1,235	393	199	778	0.22	3.2

SUMMARY.

1. The correlation between the incidence in children of dental caries, mottled enamel and fluorine content of drinking water has been studied in various parts of India.

2. A positive degree of correlation between the incidence of mottled enamel and the fluorine content of drinking water was observed which was found to be statistically significant.

3. Statistical analysis showed that degree of severity of mottled enamel was correlated with a higher concentration of fluorine.

4. A lower incidence of dental decay was recorded in 1,765 children showing mottled enamel as compared to 4,312 children showing no mottling of enamel.

5. The severity of mottled enamel had no influence on the caries incidence.

6. In a group of 1,074 healthy adults, who were not living throughout their life in an endemic fluorosis area, no association between the incidence of dental caries and mottled enamel was elicited.

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ERUPTION AGE OF TEETH IN INDIA.

BY

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As far as we are aware no attempt has so far been made to estimate with precision the age of eruption of the different teeth in any part of India. The only study of this subject appears to be that of Powell (1902) in Bombay, but his work was an attempt to define the range of variation rather than to estimate the mean age of eruption. Even in the advanced countries of the West very little attention has yet been paid to determine with a desirable degree of accuracy, the age at which the teeth erupt in different sections of the population. Klein, Palmer and Kramer (1937) seem to be the first to have recognized the need of precise assessment of eruption age and claim to have developed a suitable statistical technique for interpreting their data. Previous workers, viz. Suk (1919), Magitot (1883), Broca (1879), Mayet (1912), Cherot (1912), Welcker (1886), and Livy (1879), adopted methods of estimation which according to Klein *et al.* (*loc. cit.*) are not statistically sound.

The problem is of medico-legal importance because in cases of uncertainty regarding the true age of children and adolescents the condition of the teeth is one of the chief criteria used in making an estimation. This is true in India more than elsewhere because little reliance can be placed on individual birth records or on the average person's statement concerning his or her age.

To a student of dental diseases also a knowledge of the age of tooth eruption is necessary in order to correlate disease incidence with the post-eruptive age of the teeth.

Relevant data for the estimation of the eruption age of teeth reported in this paper were collected (i) from the rice-eating areas (Madras, South India) and (ii) from the wheat-eating areas (Lahore, North India). The boys and girls examined were from different schools and their ages were determined from the dates of birth recorded in the school registers. For each child a card was prepared on which the presence or absence of each permanent tooth was recorded. The total numbers of boys and girls of all ages, ranging from 5 to 21, examined were as follows:—

			Number examined.	
			Boys.	Girls.
Rice-eating area (Madras City)	1,412	474
Wheat-eating area (Lahore City)	1,713	...

In Tables I to III figures are set out separately for South Indian boys and girls and for boys in North India. The percentage of children showing the presence of any particular permanent tooth can also be read out for each age in these tables.

In each case, at the lower ages, the percentages are zero while at the higher ages they all reach 100. If these percentages are charted against age they follow the shape of the so-called sigmoid curve.

Different types of mathematical equations have been used to describe these curves. A group of English investigators have fitted what is called the logistic curve. Klein *et al.* (*loc. cit.*) used the equation of the normal curve of error for this purpose as well as to describe the eruption process. Although both the methods involve heavy computational work, the use of the normal curve has the advantage that along with an estimate of the average age a few other constants, e.g. the standard error of each estimate, the percentage of children below any age in whose mouths teeth have erupted, the proportion of teeth erupting between any two ages, as also the velocity of eruption, can be expressed numerically.

TABLE I.

Percentage of children with permanent teeth at each age.
South Indian boys.

Age.	Total number examined.	Central incisor.				Lateral incisor.				Canine.				1st bicuspid.			
		U. J.		L. J.		U. J.		L. J.		U. J.		L. J.		U. J.		L. J.	
		L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.
6	7	64	14	14	14
7	11	97	64	64	82	36	27	36	45	9
8	31	100	94	100	100	55	58	81	87	16	10	3	6	7	10	3	6
9	58	100	100	100	100	88	83	91	93	19	16	20	24	24	20	17	26
10	101	100	100	100	100	93	95	98	98	33	33	40	38	46	51	42	34
11	137	100	100	100	100	99	99	100	100	55	51	72	70	70	74	56	72
12	146	100	100	100	100	99	99	100	100	84	77	86	86	91	94	82	84
13	174	100	100	100	100	100	99	100	100	95	93	98	99	98	99	97	97
14	200	100	100	100	100	100	100	100	100	93	95	98	97	96	96	92	95
15	169	100	100	100	100	100	100	100	100	99	99	99	99	100	98	99	99
16	143	100	100	100	100	100	100	100	100	99	100	100	100	100	100	100	100
17	96	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
18	64	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
19	38	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
20	14	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
21	15	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

U. J. = Upper jaw ; L. J. = Lower jaw ; L. = Left ; R. = Right.

TABLE I—*concl.*

Age.	Total number examined.	2nd bicuspids.				1st molar.				2nd molar.				3rd molar.			
		U. J.		L. J.		U. J.		L. J.		U. J.		L. J.		U. J.		L. J.	
		L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.
6	7	43	43	43	43
7	11	91	98	98	98	3	3	6	9
8	31	3	3	100	100	100	100	9	9	10	19
9	58	16	14	16	17	100	100	100	100	11	10	26	31
10	101	23	23	23	18	100	100	100	100	27	26	47	64
11	137	52	44	44	44	100	100	100	100	79	80	86	95	1	1	2	2
12	146	77	70	70	63	100	100	100	100	91	91	91	95	2	2	3	7
13	174	90	91	91	87	100	100	100	100	100	100	99	100	4	3	5	12
14	200	92	93	93	95	100	100	100	100	100	100	99	99	6	6	14	21
15	169	99	99	99	99	100	100	100	100	100	100	100	100	18	19	31	30
16	143	100	100	100	100	100	100	100	100	100	100	100	100	19	19	39	34
17	96	100	100	100	100	100	100	100	100	100	100	100	100	100	100	71	71
18	61	100	100	100	100	100	100	100	100	100	100	100	100	79	93	53	80
19	38	100	100	100	100	100	100	100	100	100	100	100	100	100	100
20	14	100	100	100	100	100	100	100	100	100	100	100	100
21	15	100	100	100	100	100	100	100	100	100	100	100	100

U. J. = Upper jaw; L. J. = Lower jaw; L. = Left; R. = Right.

TABLE II.

Percentage of children with permanent teeth at each age.
 South Indian girls.

Age.	Total number examined.	Central incisor.						Lateral incisor.						Canine.						1st bicuspid.					
		U. J.			L. J.			U. J.			L. J.			U. J.			L. J.			U. J.			L. J.		
		L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	L.	R.	L.	L.	R.	L.	L.	R.	R.
6	5	20	20	20	20	20	20	20	20	20	20	20	20
7	9	67	67	67	67	56	56	67	67	67	73	73	73
8	15	93	87	87	93	93	40	80	80	56	56	56	56
9	28	96	96	100	100	93	89	96	96	100	100	100	100	11	14	21	21	29	29	21	25	14	14	7	7
10	34	100	100	100	100	97	100	100	100	100	100	100	100	35	35	53	53	50	50	44	47	41	41	35	35
11	54	100	100	100	100	100	98	93	93	100	100	100	100	78	65	81	81	81	81	61	78	78	78	79	79
12	50	100	100	100	100	100	98	100	100	100	100	100	100	94	94	98	98	98	98	94	100	100	100	98	98
13	50	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
14	41	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
15	41	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
16	46	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
17	33	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
18	21	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
19	14	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
20	8	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
21	2	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

U. J. = Upper jaw; L. J. = Lower jaw; L. = Left; R. = Right.

TABLE II—*concl'd.*

Age.	Total number examined.	2nd bicuspid.						1st molar.						2nd molar.						3rd molar.					
		U. J.			L. J.			U. J.			L. J.			U. J.			L. J.			U. J.			L. J.		
		L.	R.	L.	L.	R.	L.	L.	R.	L.	L.	R.	L.	L.	R.	L.	L.	R.	L.	L.	R.	L.	L.	R.	L.
6	5	20	20	40	20
7	9	89	89	89	89
8	15	7	100	100	100	100
9	28	7	4	7	4	100	100	100	100	100	4	4	4	4	4	4	4	4	4
10	34	32	18	18	18	49	100	100	100	100	9	12	18	21
11	54	49	49	48	49	82	100	100	100	100	32	39	50	36
12	50	82	84	86	82	96	100	100	100	100	74	74	84	86
13	50	96	92	96	96	98	100	100	100	100	92	90	96	94	4	4	4	4	4
14	41	95	98	98	100	100	100	100	100	100	93	95	100	100	100	100	100	100	100	5
15	41	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	2	2	2	2	2	2
16	40	93	100	100	98	100	100	100	100	100	93	100	100	100	100	100	100	100	100	13	9	15	15	15	15
17	33	97	97	100	97	100	100	100	100	100	96	96	100	100	100	100	100	100	100	24	24	27	21	21	21
18	21	100	95	95	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	5	10	20	15	15	15
19	14	100	100	100	100	100	100	100	100	100	94	94	100	100	100	100	100	100	7	14	21	21	21	21	21
20	8	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	36	38	38	38	38	38	38
21	2	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

U. J. = Upper jaw ; L. J. = Lower jaw ; L. = Left ; R. = Right.

TABLE III.

Percentage of children with permanent teeth at each age.
North Indian boys.

Age.	Total number examined.	Central incisor.				Lateral incisor.				Canine.				1st bicuspid.			
		U. J.		L. J.		U. J.		L. J.		U. J.		L. J.		U. J.		L. J.	
		L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.
6	36	39	33	55	50
7	20	80	90	70	75	35	10	55	55
8	59	97	93	98	95	57	39	78	52	13	25	22	13	15	10	18	18
9	44	95	97	98	100	79	68	81	79	29	27	29	38	9	34	34	34
10	58	100	100	100	100	100	100	100	100	31	34	44	53	53	44	51	51
11	107	100	99	99	99	99	98	99	99	65	59	73	76	78	69	72	72
12	216	100	100	100	100	100	98	100	100	88	88	91	93	95	94	90	92
13	267	100	100	100	100	100	100	100	100	93	93	97	97	97	96	96	96
14	355	100	100	99	100	99	100	100	100	98	99	99	99	99	99	99	99
15	313	100	99	99	100	100	100	100	100	99	99	99	99	99	99	99	99
16	145	100	100	100	100	100	100	100	100	99	99	99	99	99	99	99	99
17	57	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
18	18	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
19	4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
20	3	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
21	3	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

U. J. = Upper jaw; L. J. = Lower jaw; L. = Left; R. = Right.

TABLE III—*concl'd.*

Age.	Total number examined.	2nd biousspid.						* 1st molar.						2nd molar.						3rd molar.					
		U. J.			L. J.			U. J.			L. J.			U. J.			L. J.			U. J.			L. J.		
		L.			R.			L.			R.			L.			R.			L.			R.		
		L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.	L.	R.		
6	36	75	63	72	63		
7	20	95	95	85	95		
8	59	15	15	3	12	100	100	100	98	100	100	4	3	3	3		
9	44	16	18	20	23	100	100	100	100	100	100	10	7	16	16		
10	58	27	34	41	27	100	100	100	100	100	100	24	24	41	33		
11	107	57	57	41	45	100	100	100	100	100	100	36	33	42	42		
12	216	86	78	75	73	100	100	100	100	100	100	78	66	75	71		
13	207	91	92	86	86	100	100	100	100	100	100	87	79	98	97		
14	355	98	97	95	96	100	100	100	100	100	100	95	95	95	95	1		
15	313	98	99	98	98	100	100	100	100	100	100	96	95	97	97	2	0.5	3	3		
16	145	100	98	99	98	100	100	100	100	100	100	100	100	100	100	1.5	0.6	5	7		
17	57	100	100	100	100	100	100	100	100	100	100	100	98	99	100	11	0.6	14	17		
18	18	100	100	88	100	100	100	100	100	100	100	94	100	100	89	5.5	11	22	16		
19	4	100	100	100	100	100	100	100	100	100	100	100	100	100	100	25	25	25	25		
20	3	100	100	100	100	100	100	100	100	100	100	100	100	100	100		
21	3	100	100	100	100	100	100	100	100	100	100	100	100	100	100		

U. J. = Upper jaw; L. J. = Lower jaw; L. = Left; R. = Right.

The question as to which type of curve will adequately describe the eruption process must still be considered to be controversial. It is not proposed in this paper to discuss the purely theoretical aspect of these mathematical attempts at giving expression to the eruption process of teeth. Suffice it to state that whilst we agree with Klein *et al.* (*loc. cit.*) that the application of the normal curve of error has several advantages, we are not at all convinced that this curve could be applied to express the eruption of each tooth separately. Klein *et al.* (*loc. cit.*) do not seem to have tested whether this curve can be used satisfactorily for each tooth separately. The findings of the writer, as explained in a subsequent paragraph, are that frequency curves of a variety of shapes are met with and that it would be futile to adhere to a curve of rigid shape like the normal curve which does not permit of sufficient adaptability to such data.

In the three Charts are presented histograms, one for each tooth. The height of the bar against each age indicates the percentage of children in whom teeth erupted at that particular age. Data for all the 32 teeth have been charted together, eight in a column for purposes of comparison.

The histogram for the first molar follows definitely the usual J-shaped form. The eruption of this tooth is completed within three years, viz. between the ages of 6 and 8 years. If a true form of curve for this tooth is to be determined it will be necessary to record data on smaller intervals of say a month on a very much larger number of children. For practical purposes, however, the diagram now shown indicates clearly that in the majority of children this tooth erupts at the age of 7 years.

Histograms of teeth which erupt later show that there is a tendency for the shape to become symmetrical and only in the case of the two bicuspid and of the canine can it be accepted that the normal curve would be satisfactory. Records of the latest teeth to erupt show that there is again a tendency for the histogram to become asymmetrical but the skewness is of the negative type. For instance the eruption of the third molar is relatively rare before the age of 20 to 21 years by which age this tooth has almost invariably erupted. For these extreme types of skew curves we cannot at this stage suggest any single definite form of mathematical equation. This, however, is merely of academic interest. For practical purposes what matters is the knowledge as to when the large majority of individuals have any particular teeth erupted. This information is sufficiently indicated by the histograms.

From the information set out in Tables I to III estimates have been made of the average ages of eruption for each tooth. These are given in Table IV separately for South Indian boys and girls and for Lahore boys.

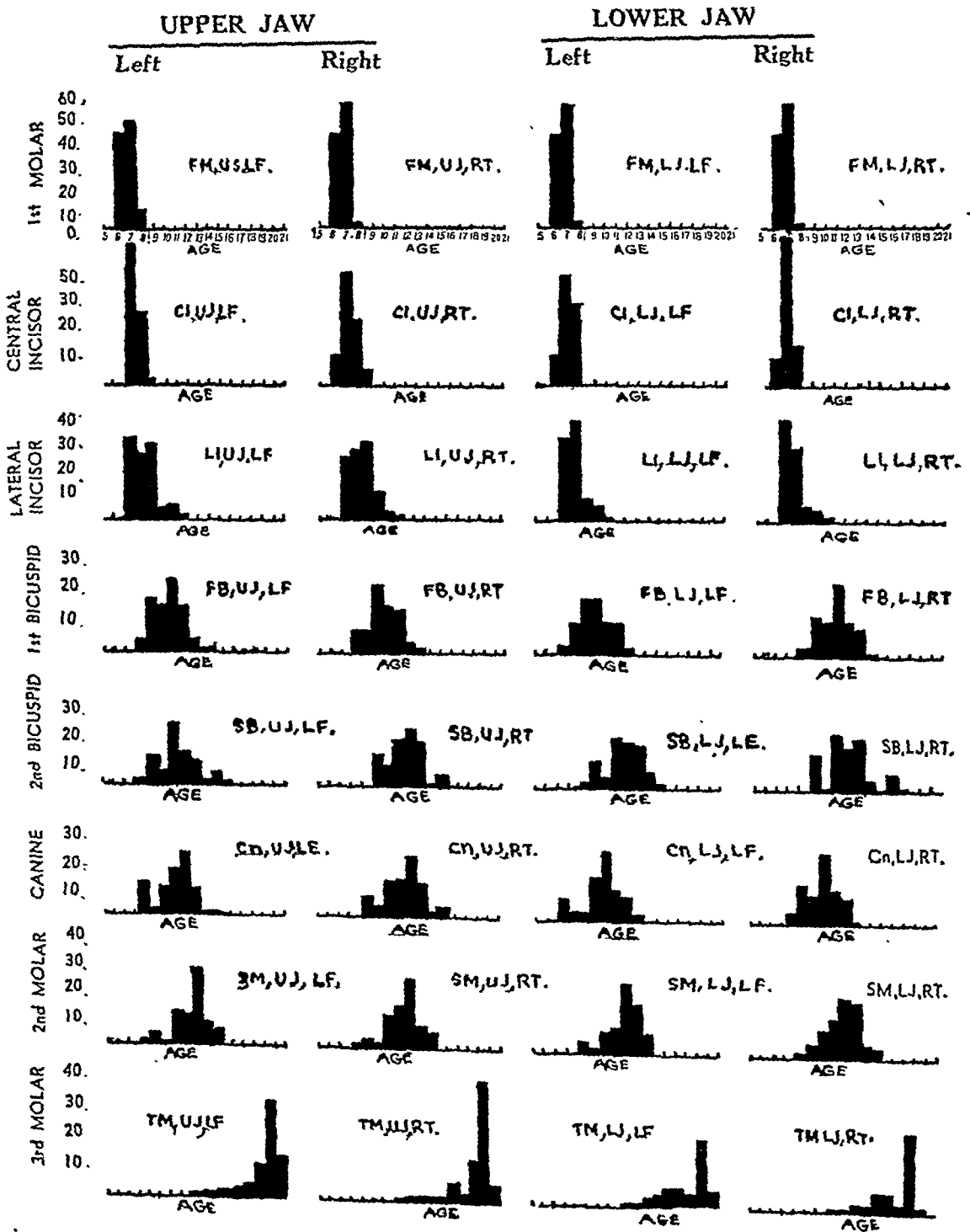
An explanation is necessary in regard to the age shown against each group. As already stated, this information was taken from the school registers containing dates of birth and, from this, the nearest age to the month was noted. When thus recording the age, months exceeding 6 were taken as another extra year and those less than 6 were rejected. The age was thus recorded in the nearest whole number according to the birth date. The date of birth is not always recorded with perfect accuracy in this country and may in exceptional instances be in error to the extent of a year or more. In discussing small differences of fractions of a year this point has to be borne in mind.

The first molar is the earliest permanent tooth to erupt; then follow central incisor, lateral incisor, first bicuspid, canine, second bicuspid and the second and third molars. The mean figures fail to show any consistent difference between boys and girls in South India or between the two groups of boys in South India and in Lahore (Punjab). However, a few points of interest may be mentioned:—

- (i) The first molar and the central incisor seem to erupt earlier among boys than girls.
- (ii) The lateral incisor, first bicuspid, and canine erupt earlier amongst girls.
- (iii) The first and second molars, the central incisor and the canine erupt earlier in Lahore boys than in Madras boys.
- (iv) The second bicuspid, on the other hand, erupts earlier amongst Madras boys than Lahore boys. The same is generally true of the lateral incisor also.

CHART 1.

Histograms showing for South Indian boys the percentage of teeth erupting at each age.



CODES: UJ—Upper jaw, LJ—Lower jaw, LF—Left, RT—Right, FM—1st molar, CI—Central incisor, LI—Lateral incisor, FB—1st bicuspid, SB—2nd bicuspid, Cn—Canine, SM—2nd molar, TM—3rd molar.

Histograms showing for South Indian girls the percentage of teeth erupting at each age.

UPPER JAW

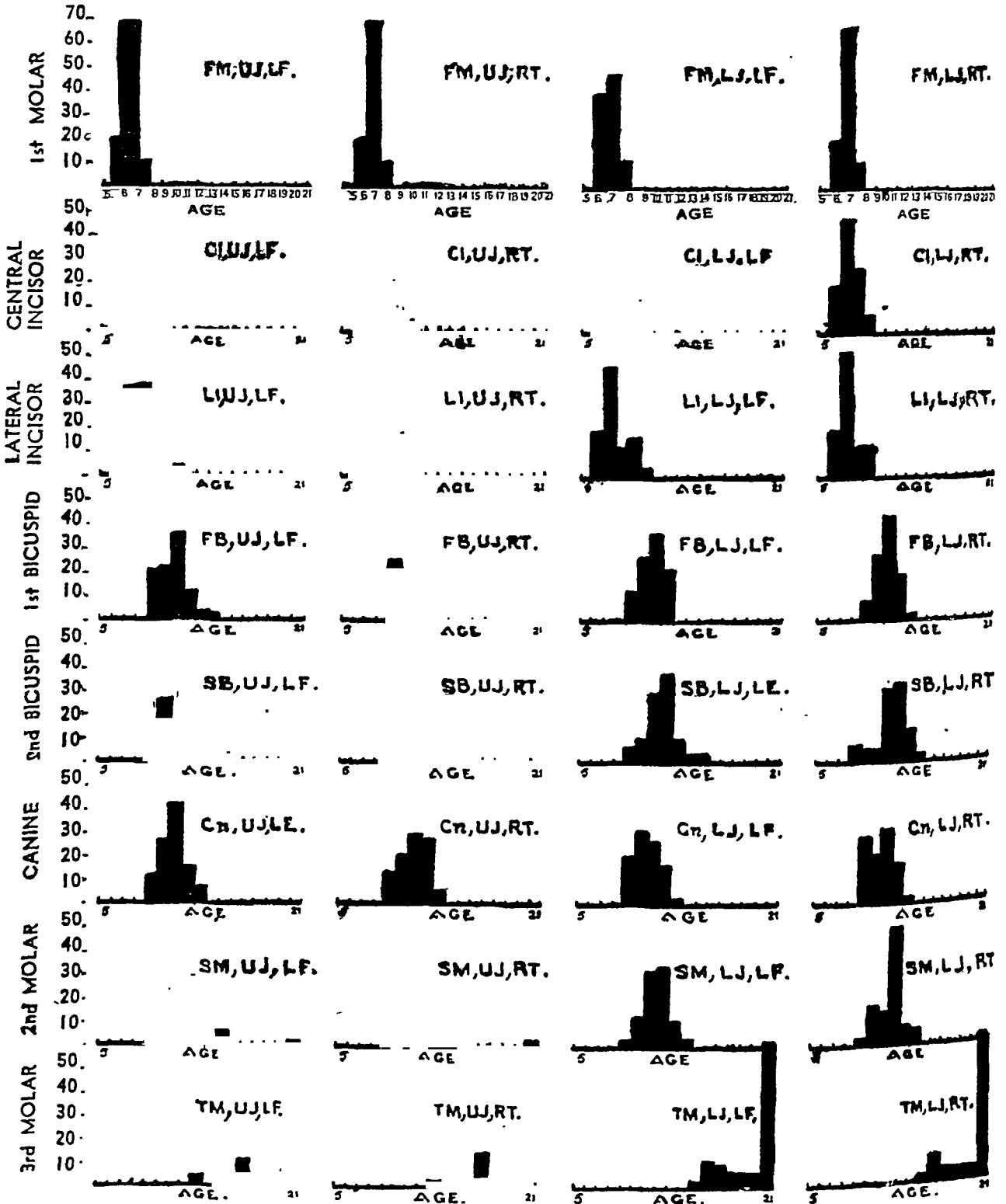
LOWER JAW

Left

Right

Left

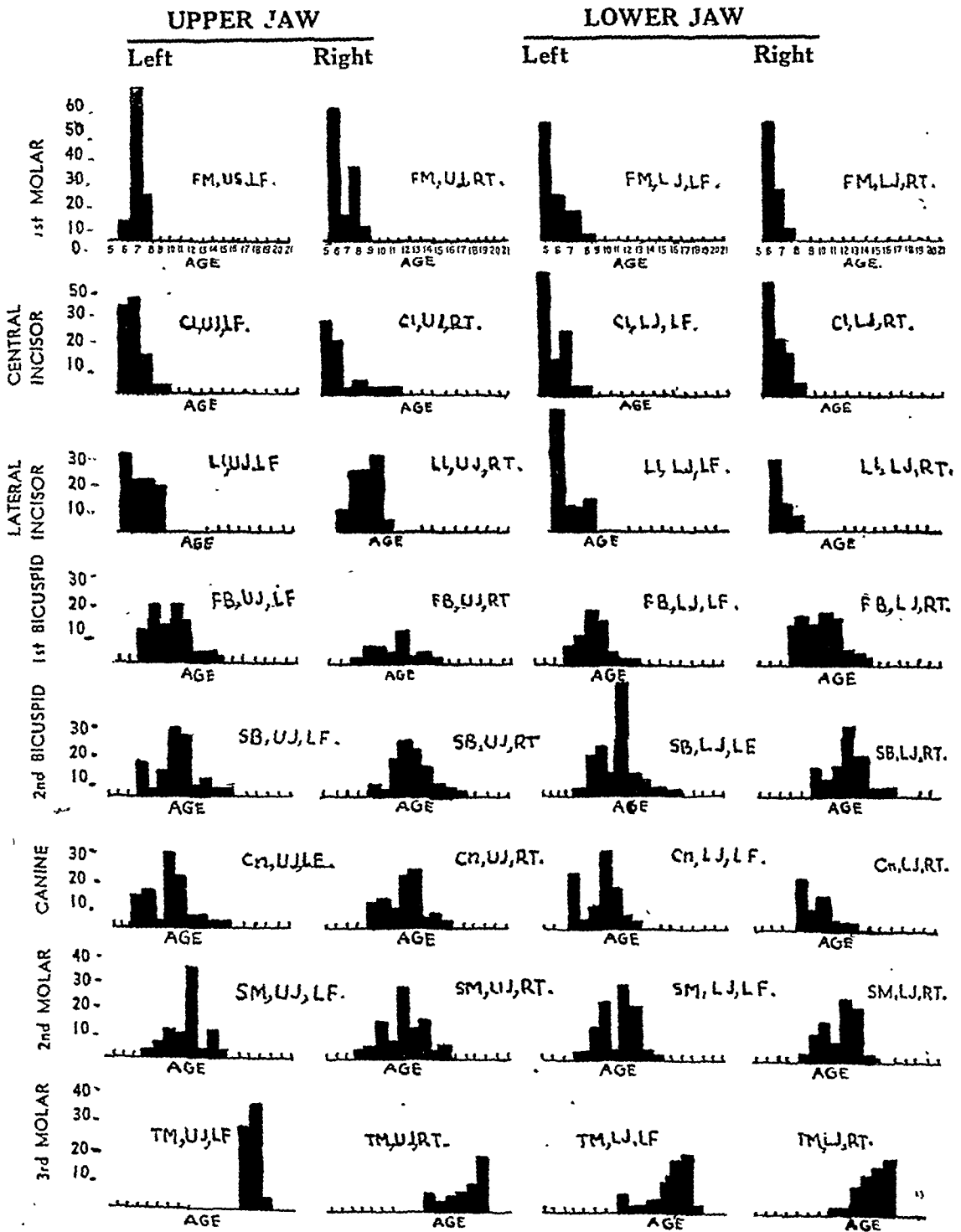
Right



CODES: UJ—Upper jaw, LJ—Lower jaw, LF—Left, RT—Right, FM—1st molar, CI—Central incisor, LI—Lateral incisor, FB—1st bicuspid, SB—2nd bicuspid, Cn—Canine, SM—2nd molar, TM—3rd molar.

CHART 3.

Histograms showing for Lahore boys the percentage of teeth erupting at each age.



CODES: UJ—Upper jaw, LJ—Lower jaw, LF—Left, RT—Right, FM—1st molar, CI—Central incisor, LI—Lateral incisor, FB—1st bicuspid, SB—2nd bicuspid, Cn—Canine, SM—2nd molar, TM—3rd molar,

TABLE IV.

The mean ages of eruption of different teeth in South Indian boys and girls and in North Indian boys.

Tooth.	Jaw.	Side.	South Indian girls (years).	South Indian boys (years).	Lahore boys (years).
1st molar	Upper	Right ...	6.91	6.59	5.90
		Left ...	6.91	6.66	6.21
	Lower	Right ...	6.91	6.59	5.90
		Left ...	6.71	6.59	5.93
Central incisor	Upper	Right ...	7.30	7.28	6.87
		Left ...	7.24	7.39	6.87
	Lower	Right ...	7.20	7.04	6.80
		Left ...	7.26	7.22	6.78
Lateral incisor	Upper	Right ...	7.62	8.38	8.83
		Left ...	7.39	8.29	8.29
	Lower	Right ...	7.70	7.77	9.16
		Left ...	7.37	7.94	7.06
1st bicuspid	Upper	Right ...	10.50	10.52	10.60
		Left ...	10.60	10.66	10.27
	Lower	Right ...	9.46	10.79	10.38
		Left ...	10.67	10.96	10.58
2nd bicuspid	Upper	Right ...	11.57	10.66	11.10
		Left ...	11.36	10.38	11.11
	Lower	Right ...	11.36	11.80	12.44
		Left ...	11.47	11.71	13.92
Canine	Upper	Right ...	10.92	11.26	11.19
		Left ...	10.82	11.00	10.84
	Lower	Right ...	10.42	10.77	10.40
		Left ...	10.62	11.66	10.50

TABLE IV—*concl'd.*

Tooth.	Jaw.	Side.	South Indian girls (years).	South Indian boys (years).	Lahore boys (years).	
2nd molar	{ Upper	{ Right	11·86	12·37	11·95
		{ Left	11·96	12·37	11·12
	{ Lower	{ Right	11·59	12·26	11·41
		{ Left	11·48	11·90	11·26

We have combined the eruption ages for boys and girls of both the places as well as for the four quadrants into one estimate. These mean ages of eruption are shown in Table V:—

TABLE V.

Mean ages of eruption.

Tooth.	YEARS.	
	India.	U.S.A. (Klein <i>et al.</i>)
Central incisor	... 7·10	7·34
Lateral „	... 7·88	8·38
Canine 10·87	11·42
1st bicuspid	... 10·50	10·21
2nd „	... 11·57	11·00
1st molar 6·48	6·59
2nd „ 11·79	12·55

The Indian figures are similar to those of the United States of America, except that there is however an indication that the eruption of teeth takes place somewhat earlier in India than in the United States.

We would suggest that data bearing on the problem of tooth eruption age should be collected in India over a larger population and separately for the different population groups. If these are recorded over intervals of age shorter than a year and if a large enough series is available it would be possible to calculate precisely not only the mean age of eruption of each tooth but also the range of variation and to determine the curve of eruption process. We make no claim that the estimates made by us are applicable to the whole of India.

SUMMARY.

An attempt has been made to estimate the eruption age of teeth in certain Indian populations. From the data collected in North and South India estimates have been provided for the eruption age of each tooth.

The differences noticed in the eruption ages between boys and girls are not very marked ; those between boys of South and North India are also small. Our estimates seem to indicate that in India the eruption of teeth takes place somewhat earlier than in the United States of America.

I am greatly indebted to Mr. Satya Swaroop of Punjab Public Health Department, Lahore, for the interest he has shown in the work and for the advice and the assistance he has given me.

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ANÆMIA AND MALNUTRITION IN INDIAN ARMY RECRUITS.

BY

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IN May 1945, the Anæmia Investigation Team and a Nutrition Team, General Headquarters, India, began a combined experiment on South Indian army recruits, and this paper describes the initial blood findings on the 800 subjects. The nutritional observations were entirely the work of one of the authors (O. P. V.), whilst the other three are responsible for the laboratory work and the statistical analysis.

MATERIAL.

Eight hundred and one newly-joined South Indian Pioneer recruits were examined during May and June 1945 in Harihar, Mysore State, which is 1,700 feet above sea-level. The temperature range at this time was about 80°F. to 90°F.

The majority of these men were Tamils and Telegus from the south-east and north-east parts of the Madras Presidency respectively, and Malayalis from the south-west coast. All belonged to the agricultural and labouring classes. Nearly all gave their ages as between 18 and 30 years, and most appeared to be between 18 and 20 years of age.

The economic status of these men had been very low. An investigation by one of us (O. P. V.) showed that the estimated family income barely sufficed to buy an adequate diet at pre-war prices, and during the war the cost of all necessities has risen greatly. Rice was the staple food. They ate very little animal protein; many could not afford any meat, and fish was not often available. The average consumption of milk certainly did not exceed two or three ounces daily. The main vegetable source of protein was a small amount of dhal (pulses). The fat intake was very small.

The Malayalis came from the most fertile area, and the Telegus from the least fertile, and this difference was clearly reflected both in the estimated daily diet and in the degree of malnutrition observed in the different races.

TECHNIQUE.

We determined the hæmoglobin level, red cell count, and packed cell volume of each recruit, and examined a stool for hookworm ova. Venous blood was taken at 08.30 hours, when the men had sat down for half an hour after their breakfast of tea and chapatties.

Blood.—Blood was drawn from an arm vein. No tourniquet was used, but if necessary the veins were made prominent by the brief pressure of a hand. Syringes were sterilized by hot liquid paraffin, and Wintrobe's dry oxalate mixture was used as anti-coagulant (Whitby and Britton, 1942).

Hæmoglobinometer.—Hæmoglobin was estimated as acid hæmatin matched against the glass wedge standard of the Zeiss 'Hæmometer'. In order to minimize errors due to temperature variations the machine was re-calibrated to a maturation time of eighty minutes, using bloods of known hæmoglobin content determined as alkaline hæmatin with a standard made from pure crystalline hæmin (King *et al.*, 1944). The standard error of a single reading of this instrument is ± 0.16 g. hæmoglobin.

Red cell counts.—We used Hellige red cell counting pipettes. Two Zeiss Thoma counting chambers were charged from the same pipette, and about 500 cells counted in each. If the totals for the two chambers differed by more than ten per cent another pipette was filled and the count repeated.

Hæmatocrits.—For packed cell volume determinations blood was centrifuged for 45 minutes at 3,000 r.p.m. in Wintrobe hæmatocrits.

Hookworm examinations.—We examined stools for hookworm ova by the usual gravity flotation technique in which about 1 g. of fæces is emulsified in 10 ml. of saturated saline. We counted the number of ova per microscope field (1/3" objective and $\times 6$ ocular) and roughly classified the infestation as:—

Very light	One ovum per six or more fields.
Light	One ovum per two to five fields.
Moderate	One to four ova per field.
Heavy	Five or more ova per field.

Statistical analysis.—The statistical methods used are fundamentally those of Fisher (1944). Differences between proportions were tested by the χ -squared test, and differences between means by the analysis of variance and the 't' test. When there was a possibility of interaction in a multiple classification Yates' (1934) method for the analysis of unorthogonal data was used.

RESULTS.

The hæmoglobin distribution of these recruits is summarized in Table I. Twenty per cent of Malayalis, 30 per cent of Tamils, and 40 per cent of Telegus were anæmic (hæmoglobin less than 14 g. per 100 ml.). When concomitant allowance was made for the differing incidences of malnutrition (*see below*), the mean hæmoglobin level of the Telegus was still significantly lower than that of the other races.

TABLE I.

The hæmoglobin distribution in recruits of various races.

Race.	Hæmoglobin in grammes per 100 ml.												Total.	Mean.	S. D.	
	6-	7-	8-	9-	10-	11-	12-	13-	14-	15-	16-	17-				
Tamil	...	1	...	1	4	2	15	19	69	101	126	66	6	410	14·66	1·470
Malayalam	1	3	8	7	25	71	83	25	8	231	14·75	1·350
Telegu	...	1	1	3	7	12	28	37	34	14	...	137	14·12	1·633
Canarese	2	1	...	1	3	8	2	...	17	14·37	1·885
Others	2	2	2	6	14·62	...

Of these recruits 75 per cent were Hindu, 17 per cent Christian, and 8 per cent Moham-
medan; there were no significant differences between these categories.

Malnutrition and anæmia.

The difficulty of making any quantitative assessment of malnutrition is notorious, but we recorded each recruit's height and weight, the degree of muscular development, the amount of subcutaneous fat, and any signs of vitamin deficiency. This clinical assessment of malnutrition was made quite independently of the laboratory findings by one of us (O. P. V.), who is preparing a detailed report. To prevent any prejudice clinical and laboratory workers refrained from examining one another's results until the findings of both were finally recorded in writing.

There was no correlation between height or weight and anæmia, but the hæmoglobin level was clearly correlated with the other measures of nutrition.

Muscle development.—Muscle development (the amount, not quality, of muscle present) was poor in one-third of the recruits and was only moderate in the remainder. Table II shows the hæmoglobin distribution in these two groups, and it will be seen that anæmia was considerably commoner in the men with poor muscle development. Table III shows the mean hæmoglobin values of men of various races with poor and moderate muscle development. The analysis of variance (Yates' method, *loc. cit.*) gives :—

		Degrees of freedom.	Sum of squares.	Mean square.	Variance ratio.	P.
Between races	...	3	36.026	12.009	5.72	0.001
Between muscle developments	...	1	20.000	20.000	9.57	0.002
Interaction	...	3	3.818	1.273
Within groups	...	793	1,665.569	2.1003

TABLE II.

The percentage hæmoglobin distribution in recruits with poor and moderate muscle development.

(The figures from which these percentages are calculated are given in Tables VII and VIII.)

Hæmoglobin in grammes per 100 ml.									
Muscle development.		Under 11.	11–	12–	13–	14–	15–	16 and over.	Mean.
Poor	...	5	4	6	22	26	26	11	14.23
Moderate	...	1	3	4	12	27	35	18	14.30

TABLE III.

The mean hæmoglobin (g. per 100 ml.) of men of different races with poor and moderate muscle development.

MUSCLE DEVELOPMENT.								
Race.	TOTAL.						Weight of mean.	
	POOR.		MODERATE.					
	Number.	Mean.	Number.	Mean.	Number.	Mean.		
Tamil	166	14.35	244	14.87	410	14.66	395.17
Malayalam	...	67	14.29	164	14.89	231	14.75	190.27
Telegu	54	13.75	83	14.36	137	14.12	130.86
Others	8	13.54	15	14.91	23	14.43	20.87
TOTAL	...	295	14.23	506	14.80	801	14.59	737.17
WEIGHT OF MEAN ...		97.284		179.96				

In all races the mean hæmoglobin level of men with poor muscle development was lower than that of men with moderate muscle development, and the difference was clearly significant. This analysis shows also a very significant difference between races, and 't' tests between the weighted means show that the mean hæmoglobin level of the Telegus is very significantly lower than that of Malayalis and Tamils even when allowance has been made for differing 'muscle development' distributions.

Subcutaneous fat.—The amount of subcutaneous fat was also correlated with anæmia, but in a paradoxical fashion (Table IV). In the men with poor muscle development the analysis of variance gives :—

			Degrees of freedom.	Sum of squares.	Mean square.	Variance ratio.	P.
Between groups	2	47.370	23.685	9.74	Under 0.001
Within groups	292	710.314	2.432

The men with poor subcutaneous fat had the highest mean hæmoglobin level, and those with moderate subcutaneous fat had a significantly lower level ($P : 0.003$). The men with good subcutaneous fat had a yet lower mean, significantly lower than the 'moderate' group ($P : 0.01$), and very significantly lower than the 'poor fat' group ($P : 0.001$).

TABLE IV.

*The relationship of the mean hæmoglobin (g. per 100 ml.)
to muscle and fat development.*

			MUSCLE DEVELOPMENT.			
Fat development.			POOR.		MODERATE.	
			Number.	Mean.	Number.	Mean.
Poor	191	14.47	216	14.85
Moderate	98	13.89	280	14.80
Good	6	12.20	10	13.69

The men with moderate muscle development show a similar but much less marked difference :—

			Degrees of freedom.	Sum of squares.	Mean square.	Variance ratio.	P.
Between groups	2	12.875	6.4360	3.49	0.04
Within groups	503	927.625	1.8442

Here the men with good subcutaneous fat have a mean hæmoglobin level just significantly lower than that of either of the other two groups.

Vitamin deficiency signs.—Only ocular and cutaneous signs of vitamin deficiency occurred frequently enough for us to test the correlation with anæmia. The eye signs were abnormal conjunctival pigmentation, xerosis, and abnormal conjunctival vascularity, all usually

ascribed to vitamin A deficiency. The presence or absence of abnormal conjunctival vascularity was clearly shown to have no relation to anæmia, and it is not included in the 'eye signs' in the analysis below. The skin signs were marked dryness, roughness, and loss of elasticity, progressing to scaling, 'giraffe skin', and, rarely, follicular hyperkeratosis (phrynoderma). These signs have been ascribed to vitamin A or B deficiency, but Platt (1945) has recently suggested that they are a direct result of protein deficiency.

The mean hæmoglobin levels of men with well-marked signs of these various types are shown in Table V. The incidence of these signs was nearly the same in both muscle-development groups ($P: 0.7$). The analysis of variance gives:—

	Degrees of freedom.	Sum of squares.	Mean square.	Variance ratio.	P.
Between deficiency signs ...	3	36.861	12.287	5.89	Under 0.001
Between muscle developments	1	19.771	19.771	9.48	0.002
Interaction ...	3	7.447	2.4823
Within groups ...	793	1,653.315	2.0849

The mean hæmoglobin level of men with deficiency signs of both skin and eyes was very significantly lower than that of men with no such signs ($P: 0.0001$), and significantly lower than the mean hæmoglobin levels of men with skin or eye signs only ($P: 0.03$). Men with skin signs only had a mean hæmoglobin level significantly lower than that of men with no signs ($P: 0.01$), but men with eye signs only did not differ significantly from either of these two groups. All these differences were shown equally by both muscle development groups—there was no significant interaction.

TABLE V.

The relationship of the mean hæmoglobin (g. per 100 ml.) to muscle development and vitamin deficiency signs of the skin and eyes.

MUSCLE DEVELOPMENT.								Weight of mean.
Deficiency signs.					TOTAL.			
	POOR.		MODERATE.					
	Number.	Mean.	Number.	Mean.	Number.	Mean.		
None ...	43	14.66	87	15.16	130	15.00	115.11	
Eye only	25	14.27	41	15.12	66	14.80	62.12	
Skin only	133	14.36	212	14.73	345	14.59	326.91	
Skin and eye	94	13.85	166	14.61	260	14.34	240.06	
TOTAL	295	14.23	506	14.80	801	14.59	744.20	
WEIGHT OF MEAN ...	196.53		343.16		

Further analysis did not reveal any significant differences between the contributions to anæmia of any of the signs listed above.

Hookworm infestation and anæmia.

Over 70 per cent of these recruits were infested with hookworms (Table VI). The Malayalis showed significantly more infestation than either Tamils ($P: 0.005$) or Telegus

(P : 0·01), but these two latter did not differ significantly from one another (P : 0·15). The amount and degree of infestation was very nearly the same in men with poor and moderate muscle development, but it was only in the former group that infestation increased anæmia.

TABLE VI.
The percentage amount of hookworm infestation, in recruits of different races.

Race.	HOOKWORM LOAD.					Number of cases.
	None.	Very light.	Light.	Moderate.	Heavy.	
Tamil ...	26	35	19	17	3	410
Malayali ...	18	30	20	24	8	231
Telegu ...	32	31	12	19	6	137

Table VII shows the hæmoglobin distribution in recruits with poor muscle development and different hookworm loads. The incidence of anæmia (hæmoglobin less than 14 g. per 100 ml.) increased progressively from 32 per cent in non-infested men to 47 per cent in heavily infested men. Analysis of the variance of these hæmoglobin values gives:—

		Degrees of freedom.	Sum of squares.	Mean square.	Variance ratio.	P.
Between groups	4	26·129	6·5323	2·59	0·04
Within groups	290	731·555	2·5226

Significant differences are thus present between the mean hæmoglobin levels of some of these groups, and ‘t’ tests show that the means of men with light and heavy infestations are significantly lower than the means of non-infested and very lightly infested men.

TABLE VII.
The hæmoglobin distribution in recruits with poor muscle development and different hookworm loads.
Poor muscle development.

Hookworm load.	Hæmoglobin in grammes per 100 ml.													Total.	Mean.	S. D.
	6-	7-	8-	9-	10-	11-	12-	13-	14-	15-	16-	17-				
None	2	2	4	18	22	25	8	1	82	14·50	1·383	
Very light	2	1	5	7	17	25	28	13	1	99	14·40	1·572	
Light	1	...	2	3	3	14	18	10	2	...	53	13·91	1·544	
Moderate	2	3	4	15	8	10	4	...	46	14·03	1·522	
Heavy	...	1	2	...	1	2	4	4	1	...	15	13·41	2·749	
TOTAL	...	1	...	1	2	9	13	19	66	77	28	2	295	14·23	1·605	

Table VIII shows the hæmoglobin distribution in men with moderate muscle development and different hookworm loads. The analysis of variance gives :—

		Degrees of freedom.	Sum of squares.	Mean square.	Variance ratio.	P.
Between groups	...	4	15.090	3.7725	2.04	Over 0.05
Within groups	...	501	925.407	1.8471

There are thus no significant differences in the mean hæmoglobin levels of the various hookworm groups, and even the men with heavy infestation did not differ significantly from non-infested men ($P : 0.1$).

TABLE VIII.

The hæmoglobin distribution in recruits with moderate muscle development and different hookworm loads.

Moderate muscle development.

Hookworm load.	Hæmoglobin in grammes per 100 ml.													Total.	Mean.	S. D.
	6-	7-	8-	9-	10-	11-	12-	13-	14-	15-	16-	17-				
None	2	...	2	5	15	24	38	27	4	117	14.99	1.420
Very light	1	2	7	19	43	71	21	2	166	14.87	1.133
Light	2	...	8	2	8	30	23	12	2	87	14.60	1.505
Moderate	...	1	5	2	14	28	38	15	4	107	14.79	1.521
Heavy	1	3	3	12	6	4	...	29	14.53	1.297
TOTAL	...	1	4	1	18	19	59	137	176	79	12	506	14.80	1.365

Age and anæmia.

We made every effort to obtain the correct age of these men, but many were obviously considerably older or younger than they stated.

Only in the Tamils was there any correlation between the stated age and the hæmoglobin level (Table IX). Here the mean hæmoglobin level rose progressively from the 'under 19 years' group to the '23 years' group, and the analysis of variance gives :—

	Degrees of freedom.	Sum of squares.	Mean square.	Variance ratio.	P.
Between groups	7	98.233	14.033	7.19	Under 0.001
Within groups	402	784.986	1.9527

Significant differences between groups are very clearly present. The following successive differences in mean hæmoglobin level are significant: under 19 and 20 years ($P: 0.001$); 19 and 22 years ($P: 0.01$); 20 and 22 years ($P: 0.04$); 20 and 25 years ($P: 0.001$). The mean of the '24 years' group is, however, very much below that of either adjacent group (P less than 0.001), and is significantly lower than that of any group over 19 years. The 19 men of this '24 years' group were closely questioned, but all persisted in giving 24 as their age, though in appearance they seemed to range from 18 or less to over 30 years. All could count up to thirty quite fluently in their own language, and we have no idea why a group of men more anæmic than the average should have so strong a predilection for the number 24.

TABLE IX.

The mean hæmoglobin of Tamil recruits at different ages.

Age in years.		Under 19	19-	20-	21-	22-	23-	24-	25 and over.
Number of cases	...	45	30	144	23	49	22	19	78
Mean Hb. g./100 ml.	...	13.76	14.17	14.56	14.77	15.04	15.24	13.64	15.23
S. D.	...	1.624	0.995	1.327	1.687	1.272	1.286	2.567	1.110

There was no further significant change in the mean hæmoglobin level of age groups above 25 years.

We may conclude that anæmia possibly becomes less common in Tamils with increasing age from late adolescence to 22 years, but a safe opinion could only be formed if some much more reliable estimate of age than the man's own statement were available.

Splenomegaly and anæmia.

Seven recruits had a palpable spleen extending two or three finger-breadths below the costal margin. With one exception (hæmoglobin 15.6 g.) all had a hæmoglobin level of 13 g. per 100 ml. or lower, and the mean, 12.37 g., was very significantly lower than that of men without splenomegaly ($t: 4.017$). Of these men, 4 had normocytic normochromic anæmia, 1 normocytic hypochromic, and 1 microcytic normochromic. Only one (hæmoglobin 10.2 g.) was proved to have chronic malaria.

One further case had a just-palpable spleen; his hæmoglobin level was 15.1 g., and he had chronic malaria.

None of these men with a palpable spleen had bilirubinæmia or macrocytosis.

The types of anæmia present.

There were no differences between the various races or nutritional levels in regard to the types of anæmia found. Table X shows the classification of the blood counts according to hæmoglobin level, mean corpuscular volume (MCV), and mean corpuscular hæmoglobin concentration (MCHC).

TABLE X.

The relation between hæmoglobin, mean corpuscular volume and mean corpuscular hæmoglobin concentration in recruits.

MCV cu. μ .	MCHC per cent.	Hæmoglobin in grammes per 100 ml.											
		6-	7-	8-	9-	10-	11-	12-	13-	14-	15-	16-	17-
Under 80	Under 28	...	2	...	1	1	3	1
	28—	1	2	2
	30—	2	...	2	1
	32 and over	1	2	2	2	1	...
80—89	Under 28	1
	28—	4	4	4	6	1
	30—	1	7	7	28	15	7	3	1
	32 and over	5	18	53	72	27	3
90—99	Under 28	3
	28—	3	2	3	1
	30—	2	...	6	8	32	68	71	18	4
	32 and over	4	22	51	80	39	3
100 and over	Under 28	2
	28—	1	2	1
	30—	4	3	3	...
	32 and over	1	...	1	2	1	3	3

Hypochromia became progressively more common as the anæmia became more severe. Most of the milder anæmias were normochromic (MCHC 30 per cent or over), but the MCHC was usually under 32 per cent. This suggests that some iron deficiency was present, for most men with 15 g. hæmoglobin or over had an MCHC of 32 per cent or over.

Most of the anæmia was normocytic, but there was a higher proportion of microcytic anæmia in the lower hæmoglobin groups. Macrocytic anæmia was rare and mild; the most marked case had: hæmoglobin 11.9 g., MCV 120 cu. μ , MCHC 32 per cent. Of 7 macrocytic anæmias found, 5 were hypochromic. No case of macrocytic anæmia had bilirubinæmia or splenomegaly.

A few men with 14 g. hæmoglobin or over had a MCV over 100 cu. μ but under 103 cu. μ .

DISCUSSION.

No deduction can be made from this paper as to the prevalence of anæmia in the South Indian population. Our subjects were selected by recruiting officers at a time when the supply of recruits was near to exceeding the demand, and they were certainly physically above the average of their class. Both in nutrition and in blood picture they were much

superior to the Madras sweepers recruited examined by the same technique in 1944 (Hynes *et al.*, 1945).

A correlation between malnutrition and anaemia has often been inferred, but is not easily proved statistically, for it is impossible accurately to measure the degree of malnutrition. In this paper we have proved a very clear correlation between malnutrition, as assessed clinically, and anaemia, but the differences observed were relatively slight and the proof of their significance demanded an efficient statistical technique.

Anaemia was clearly correlated with muscle development. Two-thirds of the recruits had moderately good muscle development, and of these 20 per cent had less than 14 g. haemoglobin per 100 ml., and 53 per cent had 15 g. or over. There was nearly twice as much anaemia among the other third of the men, whose muscle development was poor: 37 per cent had less than 14 g. haemoglobin, and 37 per cent had 15 g. or over.

The amount of subcutaneous fat may also be taken as a measure of nutrition. Anaemia was very clearly least common in men with poor subcutaneous fat, and most common in men with good subcutaneous fat—8 out of 16 men in this latter group had less than 14 g. haemoglobin per 100 ml., and only 3 had 15 g. or over.

Since we were here dealing with men who had led an active agricultural life, it seems reasonable to assume that on the average the amount of subcutaneous fat is determined by the adequacy or otherwise of the calorie intake, whereas muscle development is dependent on a sufficiency of both calories and protein. Our findings therefore suggest that the anaemia of malnutrition is associated rather with lack of protein than with lack of calories. It also appears that anaemia is favoured by a high-calorie low-protein diet, but obviously such a hypothesis needs much more critical testing than our data afford.

There was a very clear correlation between anaemia and ocular and cutaneous signs of vitamin deficiency. It is more probable that anaemia and vitamin deficiency are independent results of malnutrition than that either influences the other. There is, however, some reason for ascribing this type of anaemia to protein deficiency, and if, as Platt (*loc. cit.*) suggests, the skin lesions have the same causation, then we have here a more direct connection.

In the more malnourished third of the men an increasing hookworm load progressively added to the amount of anaemia, but no such correlation could be demonstrated in the better-nourished men. We have here further evidence for the view that the blood loss associated with hookworm infestation is easily borne if the diet is adequate, but that infestation is a factor in causing or adding to anaemia in under-nourished men.

The very clear correlation between anaemia and slight splenomegaly may be taken as evidence that malaria also plays a part in the production of this anaemia of malnutrition.

The greater part of the anaemia was normocytic and normochromic, but in most of the milder anaemias the findings were suggestive of iron deficiency, and there was clear evidence of this deficiency in most of the severer anaemias.

Macrocytic anaemia was rare, and never of the severe type so common in hospital wards.

SUMMARY.

1. An attempt has been made to correlate the blood findings and nutritional status of 801 South Indian army recruits.

2. Anaemia was much commoner in the third of the men whose muscle development was poor than in the two-thirds whose muscle development was moderate.

3. Anaemia was least common in men with poor subcutaneous fat, and most common in men with good subcutaneous fat.

4. There was a clear correlation between anaemia and cutaneous and ocular vitamin deficiency signs.

5. In the more malnourished third of the men an increasing hookworm load progressively added to the amount of anaemia, but no such correlation could be demonstrated in the better-nourished men.

6. Anæmia and splenomegaly were correlated.

7. Most of the anæmia was normocytic and normochromic, but there was usually evidence suggestive of iron deficiency. Macrocytic anæmias were rare and mild.

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A FEEDING EXPERIMENT AMONG INDIAN ARMY RECRUITS.

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It has long been common knowledge in military circles that men recruited into the Indian army undergo a marked physical improvement during the months following enlistment, but so far as we are aware no published account of the nature and extent of this improvement exists. The chief factors likely to produce such an effect are better nutrition, physical training and control of infectious disease. Of these, improved nutrition is probably by far the most important. The average Indian recruit in time of war is, as a rule, poorly nourished when he enlists, being far more nearly representative of the impoverished general population than the relatively few highly selected men recruited in peace, and hence is likely to respond to a higher standard of feeding than that which he could obtain in his village. It is our impression that physical training, of itself, plays a secondary rôle, for the Indian agriculturalist (who forms the bulk of the army) seems to be in very good 'training' within the limitations imposed by his muscular development, which is often very poor. A hygienic environment is probably very important, especially in so far as it controls malaria and hookworm infestation, but mere eradication of such diseases is unlikely to produce a marked physical improvement unless a nutritious diet is being consumed; and, in addition, it is not possible to ensure that every man is immediately cleared of all parasites and infections likely to impair physical development.

In 1944, facilities were obtained to undertake limited studies on men in two recruit training centres in North-West India. Such centres presented unusually favourable opportunities for conducting feeding experiments under more or less controlled conditions. The average recruit was then a poor specimen, undersized and with spindly limbs, and showing many of the stigmata of specific nutritional deficiencies. One of the present authors (A. M. T.) has had considerable experience of examining children and adolescents in Britain, and is of the opinion that the majority of these recruits were physically comparable with adolescents and young men from the worst slums in that country during the years immediately preceding the war. This comparison is believed to be valid except for the relative rarity of skeletal deformities among Indians and a few differences in the regional development of muscles, reflecting the different occupations in each population. Yet these recruits were being transformed into effective troops who were to play a worthy part in subsequent campaigns under the most arduous conditions. It appeared highly desirable to study the beginnings of this process of transformation.

Many surveys conducted in India (e.g. Wilson and Widdowson, 1942; Aykroyd and Krishnan, 1937, etc.) show that the average villager subsists at a very low economic level, and consumes a diet tending to be deficient in calories and certainly deficient in most of the essential nutrients. This state of affairs is reflected in spite of careful selection in the condition of recruits volunteering for the expanded army. After enlistment, men are placed on the standard Indian army ration, which is a great improvement on the standards of consumption in villages. The actual food consumed by the subjects of our study, together with an analysis, is shown in Table I. Normally, recruits deemed to be underweight or unusually malnourished are given one pound (16 fluid ounces) of milk daily, in addition to rations; it has been considered that this will accelerate gain of weight and clinical improvement. We

have examined the effect of such extra milk by comparing two groups of recruits, one on the basic ration only, and the other on the same ration *plus* 16 fluid ounces of milk. The two groups were matched as far as possible in respect of physique, nutritional status, race, religion, environment and military function. Measurements were made of heights, weights and a selection of what were considered to be relatively objective clinical signs indicative of nutritional deficiencies; hæmatological studies were made concurrently by a team of specialists.

PROCEDURE.

The experiment was conducted in duplicate, in an infantry training centre in Abbottabad (Punjabis and Dogras) and an Indian Army Medical Corps dépôt in Rawalpindi (Punjabi ambulance sepoys, 'followers' from the United Provinces, and Madrasi sweepers). One company at each centre was designated the 'nutrition company', and arrangements were made for all men at each place to be fed from a special kitchen, and to be administered and trained, so far as possible, as a group. As new recruits arrived they were divided into two equal batches by visual matching of pairs for height, build and general physical condition. Other variables, such as race, religion and military trade were roughly matched at the same time; it was not possible to limit the groups to narrowly restricted categories and retain sufficient numbers. One batch in each centre was earmarked to receive extra milk daily, irrespective of the normal grounds for recommending such an issue (the 'milk group'), and the other, in which no man received extra milk, formed the 'control group'. Recruit drafts arrived somewhat irregularly, and it was not possible to assemble sufficient numbers to form adequately sized groups at one time. Since it was considered important to start making observations not more than one week after subjects had arrived at the centre, records had to be kept in 'staggered' fashion, the time interval between serial observations on individuals being kept as uniform as possible. The overlap between the first and last intake was of the order of 2 months, which is probably immaterial. The whole experiment was carried out between August 1944 and February 1945. A certain amount of wastage, through sickness, desertion, etc., was anticipated, but in the event was greatly underestimated, with the result that many desirable sub-groups became too small to have any analytical value and had to be either discarded or combined with other similar sub-groups.

Full records of the food supplied to and cooked in the special kitchens were kept in a daily register, and a note was made of the nature and weight of all kitchen and plate-waste deposited in swill-bins. Men of the milk group drank their extra milk at an evening parade, at which full consumption by the right men was ensured. The quality of milk supplied was checked by irregular sampling and analysis at the local military laboratory. Consumption of food from non-official sources (which is never high among Indian troops) was limited by giving the men only Rs. 3 in cash at each monthly pay day; the balance was sent to their homes under unit arrangements.

Heights were recorded to the nearest half inch, and weights to the nearest half pound. The latter were measured on a new lever-type weighing machine specially provided for the purpose, since unit weighing machines are usually worn and inaccurate. Signs of malnutrition presented many difficulties which are familiar to all who have attempted to survey objectively the nutritional status of groups. A rather extensive list of signs was at first recorded in three grades—'present', 'absent' and 'doubtful'. It was thought that with a single experienced observer (O. P. V.) conducting all examinations a fair degree of objectivity and maintenance of standards could be attained, and that bias could be further eliminated by parading the several hundred subjects randomly and recording conclusions without reference to previous findings or experimental group. A trial showed that such an expectation was not justified and that many of the serial observations on a given individual did not make sense. Eventually, records were made only of the definite presence or absence of the following conditions. Rough and dry skin; xerosis conjunctivæ; angular stomatitis; and glossitis (with fissuring). The records for these signs remained reasonably consistent throughout serial observations. Resources did not permit of biochemical studies, and it was decided for various reasons not to undertake dark-adaptation trials or slit-lamp microscopy. It did not prove useful to make notes on the general physical progress of individuals from the opinions of company officers and physical training instructors.

TABLE I.

(a) *Composition of basic rations for Indian troops.*

Official scale (daily, per head) as issued.					Amount consumed (daily, per head).				
Food.				Quantity oz.	Food.				Quantity oz.
Cereals	24·0	Atta	18·0
					Rice	4·0
<i>Vegetables and fruits.—</i>									
Onions, fresh	2·0	Onions, fresh	1·8
Potatoes, fresh	4·0	Potatoes, fresh	3·6
Dhals, dried	4·5	Dhals, dried	3·75
Other vegetables, fresh	6·0	Other vegetables, fresh, e.g. brinjals, turnips, carrots, pumpkin, cabbage, gourds, etc.	4·7
Fruit, fresh, e.g. mangoes, pears, guavas, melons, etc.	2·0	Fruit, fresh	1·5
<i>Meat and dairy produce.—</i>									
Meat, fresh, including bone	6·0	Goat flesh	3·8
Milk, fresh	6·0	Milk, fresh	6·0
Ghee	2·75	Milk ghee	1·5
					Vegetable ghee	1·1
Sugar	2·5	Sugar	2·25
Tea	0·33	Tea	0·25
Salt	0·5	Salt	0·50
Condiments	0·57	Condiments	0·50

(b) *Approximate nutritive value of the daily diet as consumed.*

Allowances for destruction of vitamins in cooking have been made.

Nutrient.		Control group.	Milk group.
Calories	3,950	4,250
Proteins (total)	...	116·0 g.	131·0 g.
Proteins (animal)	...	21·0 „	36·0 „
Carbohydrates	...	621·0 „	642·0 „
Fats	112·0	128·0
Calcium	0·84 g.	1·38 g.
Iron	54·0 mg.	55·0 mg.
Vitamin A	...	800 I.U.	1,600 I.U.
Carotene	1,800 „	1,800 „
Thiamin	3·2 mg.	3·4 mg.
Riboflavin	...	1·5 „	2·15 „
Nicotinic acid	...	29·0 „	30·0 „
Vitamin C	...	60·0 „	60·0 „

Hæmatological studies were made by the G.H.Q. Anæmia Investigation Team, commanded by Major M. Hynes, R.A.M.C., the results of which have been reported separately (see p. 119).

It remains to be stated that there are many administrative and technical difficulties in an experiment of this nature, and that we underestimated them when planning the work. The supervisory and clerical labour was very considerable and should have been handled by an officer having no other responsibilities, aided by a trained and reliable subordinate staff. So far as we were concerned, this was a counsel of perfection. The close co-operation of an expert statistician is invaluable and probably indispensable. (We did not obtain the assistance of C. K. D. until the experiment was completed.) The fact that the subjects belonged to several different religious, racial and trade categories complicated planning and the analysis of data to an undesirable degree; it would perhaps have been better to have concentrated on a relatively homogeneous group. Experimental and military requirements could not be entirely divorced from one another in the routine duties and the ultimate disposal of subjects, and often conflicted. A wastage rate of up to 20 per cent of the subjects over the first 4 months of recruit training should have been allowed for. Finally, the plan of an experiment of this nature should take into account leave schedules, religious festivals, etc., which may cause more or less serious dislocation of the routine.

ANALYSIS OF DATA.

Weights and heights.—Table II summarizes the height and weight data. Recruits who were excluded from the experiment during the period of observation are not included in the

calculations. (The causes of such exclusions were discharge on medical or disciplinary grounds, sickness other than of a minor nature, desertion and leave.)

It will be observed that the average recruit is small and light, by European standards. The Madrasi sweepers and U.P. followers formed what was undoubtedly the worst nourished group.

Effects of the milk supplement.—Increase of weight has been analysed by constructing for each subject a growth curve by the method of least squares of the type $W = A + tB$, where W is the weight at any stage of the experiment, and B is the mean increase in weight per unit of time (' t '). The mean growth rate of each group was calculated from these growth curves.

Examination of the mean increases of weight as between the milk and control groups shows that the former are higher only among the Madrasi sweepers and the Punjabi ambulance sepoys. Among other categories the reverse is the case (see Table II). The ' t ' test indicates that in no category are the differences significant. It must, therefore, be concluded that the addition of 16 fluid ounces of milk to the basic diet of these recruits did not cause any acceleration of weight gain in the period under observation.

The same result was obtained in respect of height increases. Of 485 subjects whose height records are available, only 114 showed a measurable gain. These 114 cases are distributed equally between the milk and control groups, as revealed by the χ -square test.

TABLE II.

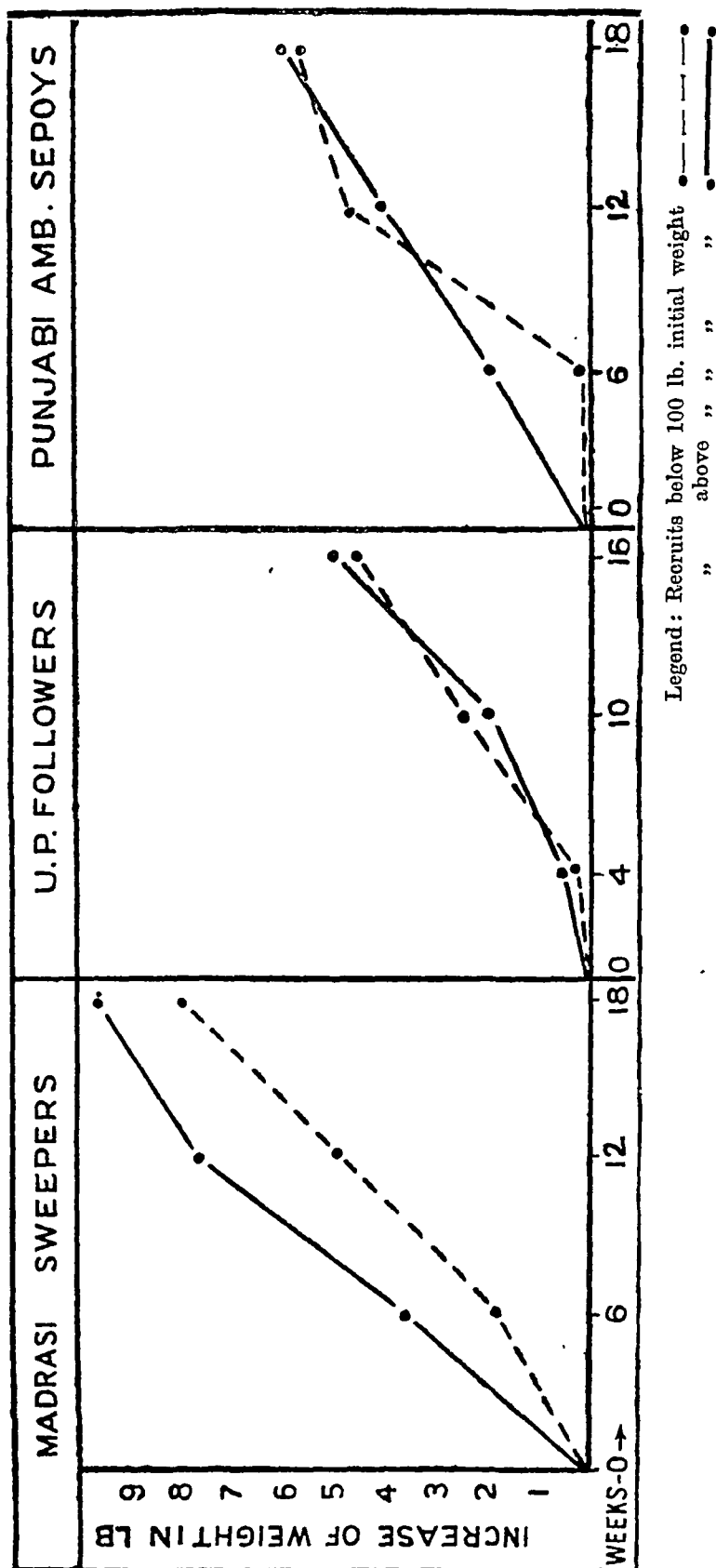
Mean heights and weights at enlistment, and mean increases in weight and the standard deviations of the variates on which the means are calculated.

Class category and period of observation (weeks).	Milk or control group.	Number of subjects.	Mean initial height (inches).	Mean initial weight (lb.).	Mean increase of weight (lb./week).
Infantry (Dogras) (20 weeks) ...	M	13	65.5±2.15	115.0±7.37	0.44±0.26
	C	17	66.0±2.81	110.0±15.02	0.47±0.26
Infantry (Punjabis) (20 weeks)	M	57	65.0±1.86	105.2±12.29	0.47±0.23
	C	38	65.3±2.22	104.5±10.02	0.48±0.27
IAMC (Punjabi ambulance sepoys) (18 weeks).	M	36	65.0±2.46	114.3±9.45	0.41±0.26
	C	41	64.9±1.90	116.8±11.00	0.29±0.31
IAMC (U.P. followers) (16 weeks)	M	26	62.6±2.44	105.9±9.51	0.27±0.31
	C	29	62.5±1.64	105.0±8.34	0.33±0.27
IAMC (Madrasi sweepers) (18 weeks).	M	13	63.1±2.79	103.1±9.12	0.49±0.27
	C	17	62.4±2.72	98.6±12.98	0.48±0.18

Nature of the increase in weight.—Graphs 1 and 2 present mean curves for the various categories of recruits; men below and above 100 lb. initial weight respectively have been separated. The weight increase tends to be greatest in the weeks immediately following enlistment. Although the rate of increase slows down with the passage of time, there is no indication that the maximum potential weight has been attained within 16 to 20 weeks. No striking differences in rate of increase between the lighter and heavier men are evident, which is confirmed on making ' t ' tests.

GRAPH I.

Increase of weight for recruits initially above and below 100 lb. in different categories.



GRAPH 2.

Increase of weight for recruits initially above and below 100 lb. in different categories.

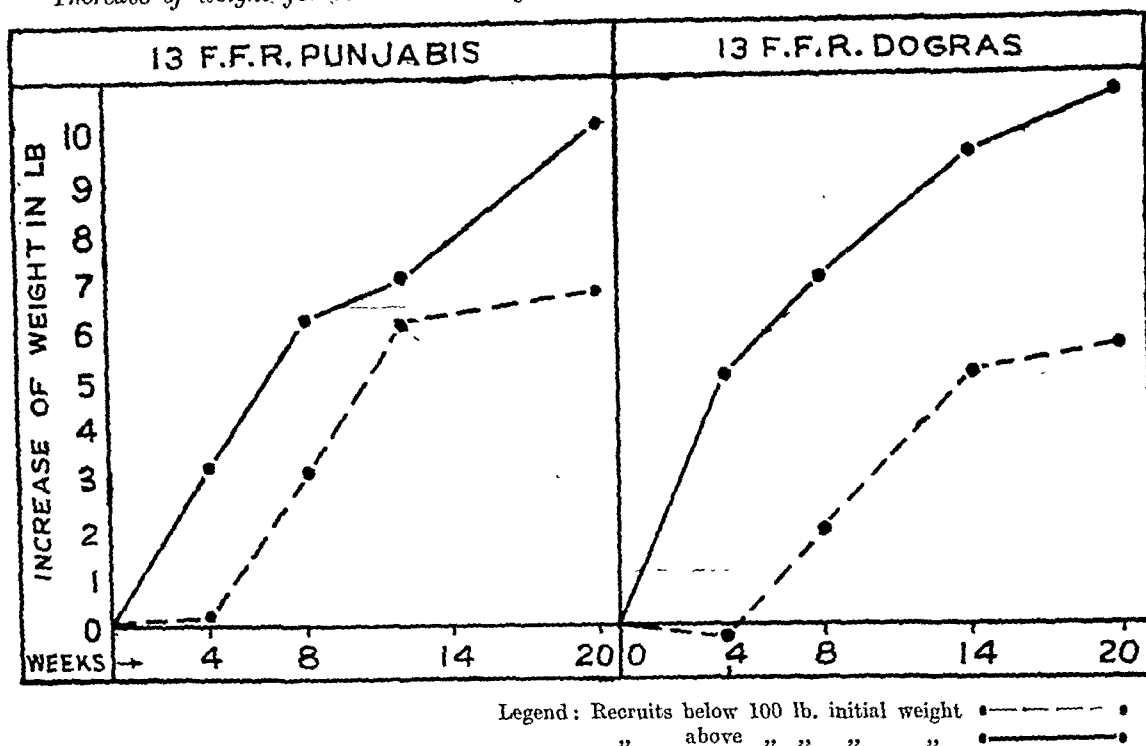


Table III separates subjects of all categories according to age, and shows the mean increase of weight in the different age groups. The mean growth rate of the 21 to 25 age group does not differ significantly from the growth rates for the other two age groups. But the growth rate for the age group of '20 years or less' differs from the growth rate of '26 years or more' at a doubtful level of significance (2 per cent) as revealed by the 't' test. Although the mean increase becomes less as age advances, yet men over 25 years of age, among whom normal growth might be expected to have ceased, nevertheless gain weight under army conditions almost as much as adolescents do. It may, therefore, be concluded that the greater part of the increase is due to environmental factors, of which improved nutrition is likely to be the most important. (It should be noted that there is rarely any precise method of knowing the real age of recruits, who themselves are usually very uncertain on the subject. In practice, we checked the man's statement at the first examination against his 'official' age, as it appeared on his enrolment form, and at the same time made an independent estimate. If there was any apparent disagreement—which was frequently the case—the man was questioned further and a 'probable age' recorded for the purposes of this investigation. A tabulation of all the probable ages in the form of a frequency distribution shows marked peaks at 18, 22, 25 and 30 years. Eighteen years is the official minimum age for enlistment, but several of our subjects appeared to be younger.)

TABLE III.

Weight increase in relation to age.

Age group, years.	Number of subjects.	Mean increase of weight (lb./week).
20 or less	210	0.425
21 to 25	55	0.389
26 or more	22	0.274

Table IV shows the correlation coefficients between initial weight and mean increase in weight for the different categories. All are negative except one, and all do not differ significantly from zero. Thus one may conclude that initial weight, *per se*, has no significant influence over weight increase.

TABLE IV.
Correlation between initial weight and increase in weight.

Category.		Number of subjects.	Correlation coefficient.
Infantry (Dogras)	30	−0·06
Infantry (Punjabis)	95	−0·06
Punjabi ambulance sepoys	77	−0·16
U.P. followers	55	−0·10
Madras sweepers	30	+0·32

Clinical progress.—Table V summarizes the findings in respect of those clinical signs for which the serial records showed a consistent standard of judgment. It shows that the signs were present to a more or less equal extent among the milk and control groups respectively at the beginning of the experiment, and that the incidence declined during the course of feeding. The χ -square test indicates that this decline was not significantly different as between the milk and control groups. The angular stomatitis cases, although improved, did not clear up completely either on milk or control diet during the course of experiment, as the riboflavin content of both diets is rather low for therapeutic purposes [see Table I (b)]. It usually takes up to 8 months for these cases to clear up completely when subsisting on the army diet.

TABLE V.
Incidence of specific clinical signs, before and after feeding.

Sign.	MILK GROUP (217 MEN).				CONTROL GROUP (223 MEN).	
		Number with sign definitely present.	Per cent.		Number with sign definitely present.	Per cent.
Rough and dry skin	{ Before	...	93	43	102	46
	{ After	...	16	7	27	12
Xerosis conjunctivæ	{ Before	...	13	6	17	8
	{ After	...	7	3	11	5
Angular stomatitis	{ Before	...	17	8	18	8
	{ After	...	17	8	16	7
Glossitis (fissured tongue).	{ Before	...	15	7	20	9
	{ After	...	8	4		5

The attempt to obtain a picture of the clinical improvement by means of such 'objective' methods has, in our opinion, failed. Table V does not give an adequate impression of the great changes manifest to ordinary clinical judgment, subjective though the latter may be. On enlistment most of these men presented a typical picture of generalized undernutrition, with many specific signs of malnutrition superadded. After 4 months in the army they were beginning to put on muscle, to carry themselves with more alertness, and to show the stigmata of malnutrition in a much less advanced degree, or in less typical form. We know, from other observations on recruits, that this improvement continues under suitable conditions for at least a year, and that at the end of that time erstwhile recruits have become healthy-looking and reliable soldiers. This is not to say that all the results of malnutrition during childhood and adolescence are wiped out, but the change is nevertheless remarkable.

We also believe that the extra milk did have an appreciable beneficial effect, in spite of lack of support from Table V. This effect manifested itself particularly in an improvement of skin texture, as a general increase in smoothness and elasticity, and as a diminution of pigmented thickening commonly found around pressure points. With the diets used the changes are slow, and vary in different individuals; the addition of milk to the basic diet appears to make them more rapid and more common. At the conclusion of our experiment, one of the authors (who had never examined individuals in association with their records, or in other circumstances which might have influenced his judgment) endeavoured to deduce which of a mixed batch of about 50 men had received extra milk, and which has not, from the condition of their skins. He selected 12 men who were considered to have received the milk, and a similar number who were thought to have received none; the remainder were classified as doubtful. Reference to the records showed that all men in the first group were in fact in the milk group; of the 'negative' group, about half the men had been wrongly allocated. This observation, though inconclusive, is considered to support the common opinion among medical officers in India that milk is a peculiarly valuable food for increasing the nutritional well-being of recruits.*

As already stated, the hæmatological data obtained by the G.H.Q. Anæmia Investigation Team have been reported separately by Major M. Hynes, R.A.M.C. For the sake of completeness, it may be mentioned here that mild hypochromic anæmia was not uncommon at the time of enlistment, and slowly decreased during the first few months of recruit training without the exhibition of anthelmintics or hæmatinics. The rate of improvement could be greatly accelerated by giving ferrous sulphate in daily doses. The extra milk had no accelerating effect. The presence and degree of anæmia could not be attributed entirely to the effects of hookworm infestation though this is undoubtedly an important factor, and a routine de-worming procedure appeared to have little practical importance. The anæmia (and possibly also the hookworm) would probably disappear in time under the ordinary nutritional and general conditions of a recruit's life, but iron, given as a medicine, is the remedy of choice. The blood of a wellnourished Indian soldier should conform to the optimum hæmatological standards for western troops, but it is possible that a semi-vegetarian diet does not provide optimum conditions for hæmopoiesis. This point is being investigated in another feeding experiment.

DISCUSSION.

The weight of a recruit, in relation to the average for his race, is perhaps the most important single expression of his potentialities as a soldier. Thus, recruiting standards prescribe minimum weights for enlistment, and the weight of individual recruits is regularly

* One of the present authors (A. M. T.) has observed a striking instance of the special value of milk in treating severe undernutrition. While visiting (with Dr. W. R. Aykroyd) several orphanages and reception camps in rural Bengal during the famine of 1943, he saw many children who had been admitted in the most advanced stages of starvation. Dehydration undoubtedly contributed towards the very marked dullness, dryness and lack of elasticity of their skins, but such a condition, perhaps in a lesser degree, is common among poorer Indian children quite apart from starvation. After as little as 3 or 4 weeks, during which they had been given a diet based on milk, these same children underwent a change almost miraculous in its extent. They became relatively plump, with glossy healthy skins, and exhibited a degree of high spirits and liveliness rarely observed among the poorer classes of India.

recorded throughout recruit training by the supervisory staffs. (We did not use the results of these routine measurements since they seldom conform to a high standard of accuracy.) The demand for man-power during the war necessitated a progressive reduction in the minimum weight standards (e.g. from 120 lb. in 1939 to 110 lb. in 1945, for Punjabi Muslims), and in addition it was permissible for recruiting officers to enrol men up to 5 lb. underweight 'provided that, in the opinion of the enrolling officer, the recruit is likely to attain the minimum regulation weight within 3 months of the date of enrolment'. Extra milk might be prescribed for underweight or undernourished recruits by unit medical officers.

Our findings indicate that the average war-time recruit can be relied on to gain 5 to 10 lb. of weight (representing about 5 to 10 per cent of his initial weight) within about 4 months of enlistment, and that this gain continues at a diminishing rate after that period. It appears to take place without much reference to age or initial weight, and is not influenced by giving 16 fluid ounces of milk in addition to the normal ration.

The effects of army life on health and well-being are generally striking to ordinary clinical observation. Many signs attributed to malnutrition regress or become modified in most cases within 4 months; others persist in relatively unchanged form. In our experience, a crude record of the presence or absence of such signs in a group of recruits does not provide an accurate index of nutritional progress, at all events within a relatively short period of observation. The prescription of extra milk is believed to accelerate clinical improvement. It is to be hoped that an experiment of this nature will be repeated with biochemical and other ancillary methods of measuring nutritional status. At present we are unable to express an opinion whether such methods will yield data accurately reflecting the subjective improvement. Meanwhile, it is considered that observation of skin texture forms the best single means of assessing nutritional status available to the ordinary clinical observer.

The basic diet which was given to these recruits is probably the most nutritious which has ever been fed to large numbers of adult Indian peasants who had never previously been subjected to planned feeding, and in our opinion it justifies the expense and organization needed to provide it. It cannot be considered an 'optimum' diet, being relatively deficient in vitamin A and in riboflavin, by the highest standards. It is possible, also, that it should contain a higher proportion of animal protein, though it remains to be seen whether the benefits commonly attributed to animal protein by physicians are not, in part, exercised by the other constituents of meat, milk, etc. Nevertheless, such a diet, whether optimal or sub-optimal, produces very striking effects on men who have practically ceased growing, and even greater benefits might be recorded were a similarly nutritious diet fed to Indian children of the peasant classes.

Our experience in this feeding experiment has led us strongly to the opinion that a diet conforming in all respects to the highest nutritional standards can be made a practical proposition so far as Indian food habits are concerned. It is too often claimed that the multifarious dietetic scruples of caste Indians, and the heavy weight of a long tradition, will prevent any effectual approach to the problem. We have found that men brought straight from their villages can be trained fairly rapidly, and with little discontent, to accept a diet differing considerably from their traditional fare. No ill effects have been observed from enforcing such changes as consumption of atta by habitual rice-eaters, and vice versa. Above all, it is our belief that the inculcation of necessary changes in dietary habits is worth the effort and cost, in that it increases capacity for work under arduous conditions in accordance with the theories of those who, for many years, have advocated diets conforming to physiological requirements.

Finally, the conditions of recruit training in Indian army regimental centres present unique facilities for feeding trials of the kind described in this paper. The administrative and technical difficulties are considerable, but if they are satisfactorily overcome, data of a very useful kind may be obtained.

SUMMARY.

1. An experimental dietary survey and feeding experiment was carried out at two Indian army recruit training centres during 1944, over a period of 16 to 20 weeks. The methods used are described.

2. A nutritious diet (the standard Indian army ration), fed to men of the Indian peasant classes, mainly aged about 20 years, caused a gain in weight amounting to about 5 to 10 per cent of their initial weight within 3 to 4 months. There was also a marked clinical improvement which, however, was not accurately reflected in a record of the incidence of certain specific signs attributed to chronic malnutrition.

3. The addition of 16 fluid ounces of milk to the basic diet did not cause any increase in the gain of weight, but is believed to have accelerated clinical improvement. The latter was manifested particularly in an improvement of skin texture.

We wish to thank the D. M. S. in India for permission to carry out this work and publish this paper, and many other officers for much encouragement and assistance. Space forbids us to mention all the latter by name, but we would thank, in particular, Major-General H. J. M. Cursetjee, C.S.I., D.S.O., K.H.S., late D.D.M.S., North-Western Army ; Brigadier F. Harris, C.B.E., M.C., late Deputy Director of Hygiene and Pathology, General Headquarters, India ; Colonel J. R. Dogra, I.M.S., Commandant, No. 1 Training Dépôt Centre, I.A.M.C. ; Colonel L. James, Commandant, 13th Frontier Force Regimental Dépôt, and Dr. W. R. Aykroyd, C.B.E., Sc.D., M.D., Honorary Consultant in Nutrition, General Headquarters, India.

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A PRELIMINARY STUDY OF THE BIOCHEMICAL CHANGES IN STARVATION CASES.*

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INTRODUCTION.

WITH the outbreak of famine in Bengal in the autumn of 1943, Calcutta and its suburbs were flooded with sick destitutes, who arrived in large numbers in search of food. Some of them, as a result of prolonged starvation, were unable to digest even the ordinary gruel, distributed from the various free kitchens and feeding centres established for this purpose. Arrangements for their admission into a special ward of the Carmichael Hospital for Tropical Diseases were made and biochemical and other studies were started before any treatment was given to them. Materials have also been collected from other destitute camps in Calcutta. Most of these patients were found to be suffering from intercurrent diseases such as malaria, dysentery, pneumonia, anæmia, etc.

Our investigation included the determination of the chemical constituents of their blood and urine. The study of carbohydrate metabolism and of the serum protein was also done in most of the cases.

I. BIOCHEMICAL CONSTITUENTS OF BLOOD.

The results of our biochemical findings are recorded in the accompanying graphs, a careful analysis of which gives the following main findings :—

- (a) Blood sugar (Hagedorn and Jensen, 1923). In the majority of the cases (70 per cent) the fasting level was definitely below the average normal, the lowest figure being 0.04 per cent (Graph 1). None of the cases, however, showed any outward symptoms of hypoglycæmia.
- (b) Non-protein nitrogen (micro-Kjeldahl method) was high in the majority of the cases (Graph 2).
- (c) Urea nitrogen (urease and Nesslerization method) was also high (Graph 3).
- (d) Creatinine (Folin and Wu, 1919) content was found to be within normal limits (Graph 4).
- (e) Uric acid (Benedict, 1922) was found to be high (Graph 5).
- (f) Cholesterol (Bloor, 1922) was low, sometimes very low (Graph 6).
- (g) Lipoid (P) (Chopra and Roy, 1936) was found to be normal in the majority of the cases. But in some instances, exceptionally high figures were obtained (Graph 7).
- (h) Plasma chloride (van Slyke, 1923) was found to be either normal or slightly lower than normal. A high chloride value was obtained only in a very small percentage of the cases (Graph 8).

* A preliminary summary of the biochemical work showing some of the results obtained was made by the senior author at a meeting held on Saturday, the 13th November, 1943, at the All-India Institute of Hygiene & Public Health, under the presidency of the D.G., I.M.S.

(i) Serum calcium (Clark and Collip, 1925) was found to be persistently low in almost all the cases (Graph 9).

II. EXAMINATION OF URINE.

Urine was examined for sugar, albumin, urea and chlorides. In spite of the fact that the carbohydrate tolerance in most of the cases was found to be lowered, sometimes to a marked degree, glycosuria was absent. Albumin was found only in traces in most of the cases, no casts or red blood cells being seen microscopically. In a few cases, however, a marked reaction for albumin was obtained and in these erythrocytes and casts were also found. None of the urine samples showed the presence of acetone bodies.

Urine urea (hypobromite method) was low. The total number of cases examined was 40 and the total excretion in 24 hours varied from 0.4 g. to 0.7 g.

Urine chloride (van Slyke, *loc. cit.*) was low (Graph 10). It should, however, be noted that low chloride excretion did not affect the plasma chloride to any appreciable degree.

III. INVESTIGATION OF CARBOHYDRATE METABOLISM.

Cases were divided into two groups according to the clinical condition as stated below :—

GROUP A: This group included cases of starvation who were usually found to be emaciated, sometimes to an extreme degree. These were generally of the 'dry' type, i.e. having no signs of œdema. Their histories revealed that the real period of starvation had started about 3 to 4 weeks earlier. Some of these were cases of 'acute' starvation, i.e. they had been practically without food during the period, while in others, living on public charity, the intake of food was uncertain. The incidence of complications in this group of cases was usually low, only a few cases having malaria and diarrhœa.

GROUP B: This group included the 'wet' cases, i.e. those with œdema. These were cases of chronic malnutrition due to starvation for prolonged periods. Their histories revealed that owing to poverty they were already on an insufficient diet prior to actual starvation; when, however, the food shortage became very acute they became indiscriminate as regards food intake and did not hesitate to eat even garbage from the streets. This might have continued for a period varying from six weeks to three months. These cases were found to have widespread œdema sometimes with marked ascites. Most of these subjects were suffering from intercurrent diseases, such as pneumonia, malaria, dysentery and anæmia. There were some cases of acute nephritis, tuberculosis, etc., amongst the destitutes admitted to hospital, but these were excluded from our present study.

Glucose tolerance test by the oral method.—Fifty grammes of glucose were given to the patients, but when this dose did not produce any change in the blood-sugar level after 3 hours, as happened in the group B cases, the test was repeated by giving 100 g. of glucose. This second test was found to give almost identical results.

Table I shows the results of glucose tolerance tests in a few patients of the B group to whom both 50 g. and 100 g. of glucose were administered at an interval of two days.

Group A cases.

The total number of cases investigated was 49. From the analysis of the results four types of curves as shown in Graph 11 were obtained. Only the average value in each type has been given.

Interpretation of the results :

Type I (Graph 11).—This is more or less a normal type of blood-sugar curve in that the initial blood-sugar level was within normal limits and the maximum rise took place within one hour after the glucose meal, the rise being well within the renal threshold level. The fall of blood sugar thereafter to the normal level took place within two hours. No glycosuria occurred.

TABLE I.

Number of cases.	Dose of glucose administered, g.	BLOOD SUGAR.					
		Before glucose.	$\frac{1}{2}$ hour after.	1 hour after.	$1\frac{1}{2}$ hours after.	2 hours after.	3 hours after.
1	50	60	65	66	66	50	46
	100	63	65	65	65	53	45
2	50	62	68	68	60	56	50
	100	64	66	67	62	58	47
3	50	65	65	65	63	55	48
	100	65	66	65	63	54	54
4	50	70	72	73	72	76	78
	100	71	72	72	72	75	79
5	50	68	70	74	72	75	75
	100	66	69	70	70	73	77
6	50	69	70	73	72	73	74
	100	68	70	73	73	75	75
7	50	64	68	70	70	74	76
	100	64	68	70	72	74	74

Type II (Graph 11).—The fasting level of blood sugar was much lower than normal. After glucose ingestion, the blood sugar rose slowly and the rise continued till the end of three hours, there being no tendency to come back to the initial level. This continued upward rise in the blood-sugar curve indicates definite delay in carbohydrate assimilation. No glycosuria occurred.

Type III (Graph 11).—The fasting level of blood sugar was normal as in type I, but the maximum rise of blood sugar after the glucose meal took place after one hour and it crossed the renal threshold level. The rate of fall of blood sugar thereafter was much slower and the return towards the initial level was much delayed. No glycosuria was present, showing that the renal threshold was higher than normal. This type of curve suggests a definite defect in carbohydrate assimilation and is similar to the curve usually obtained in cases of mild diabetes.

Type IV (Graph 11).—This is a peculiar type of curve. The fasting level of blood sugar was much below normal. The hyperglycæmic response to the ingestion of glucose was extremely abnormal. The blood sugar went much beyond the threshold limit and the rise continued for an abnormally long period. Glycosuria was observed in some cases. The nature of the blood-sugar curves suggests marked defect in carbohydrate utilization.

Group B cases.

The total number of cases investigated was 28. From the analysis of the results in this group, two main types of curves were obtained. Only the average value in each type has been given. The results are plotted in Graph 12.

Interpretation of the results :

In both types, the results were more or less of the same nature, viz. that :—

(a) The initial (fasting) level of blood sugar was low.

(b) The ingestion of 50 (or even 100) g. of glucose had little or no effect on the blood-sugar level even up to the end of three hours. In type II, however, there was a distinct tendency for the blood sugar to come down even below the initial level at the end of the second and third hours. The drop in blood sugar to such a low level as 45 mg. per cent after glucose ingestion is noteworthy.

A comparison of the above results shows that the type of blood-sugar curve were quite different in the two groups. The group B cases definitely show that there is practically no rise of the blood sugar even after the ingestion of 100 g. of glucose (Table I). In order to find out whether this peculiarity in the blood-sugar curves in this group of cases was due to defect in absorption or otherwise, glucose was given intravenously and the behaviour of the blood sugar studied every 15 minutes for one hour.

Glucose tolerance test by intravenous method (group B cases).—Glucose was administered intravenously in the proportion of 0.2 g. per kg. of the body-weight*. An analysis of the results revealed two distinct types of curves (Graph 13). It should be pointed out that these two types have no correlation with the two types of curves shown in Graph 12.

Interpretation of the results :

Type I (Graph 13).—The initial blood-sugar level was low but the blood-sugar curve after the intravenous glucose behaved almost like normal. Glycosuria was absent in all the cases.

Type II (Graph 13).—In this type the initial blood-sugar level was also low but the maximum rise of blood sugar after intravenous glucose was abnormally high (325 mg. per cent as against 190 mg. per cent in the previous type). The blood-sugar level one hour after glucose injection was still at a much higher level than before the test. Glycosuria in varying degrees was present in almost all the cases.

It will thus be seen that, though there was defective absorption in all the cases of group B, the carbohydrate utilization, as evidenced by the intravenous glucose method, was normal in some cases and markedly defective in others.

Part IV.

IV. INVESTIGATION OF SERUM PROTEIN.

On account of the difference of opinion regarding the normal levels of total serum protein albumin and globulin in Indian subjects we did a preliminary investigation on 20 healthy normal Indians. The serum protein content was estimated according to the micro-method described by Howe (1921). Our result showed that the average normal value of total serum protein of the Indians was 6.7 per cent, that of serum albumin 4.5 per cent and of globulin 2.2 per cent. These figures (Graph 14) do not differ materially from those obtained in other countries.

The investigation of serum protein was carried out on the same plan as was adopted in the case of carbohydrate metabolism. The cases were, as before, divided into two groups A and B, the results obtained in each group being tabulated separately.

* In normal healthy individuals after intravenous administration of glucose (0.2 g. per kg.) the blood sugar rises from the initial normal level (0.100 per cent) to an average level of 200 per cent within 15 to 20 minutes. The blood sugar then comes down and reaches the normal or pre-glucose level within one hour.

Group A cases.

The total number investigated under this head was 50. From a careful and critical analysis of the findings in this group, four main types of results were obtained. Only the average value in each type has been given. The results are plotted in Graph 14.

Interpretation of the results :

- (i) The total plasma protein was only slightly lower than the average normal in the first three types, but was definitely low in type IV.
- (ii) The albumin fraction was somewhat low in types I and II, and much reduced in types III and IV.
- (iii) The globulin fraction showed either normal or increased value.
- (iv) The normal albumin : globulin ratio of 2 : 1 was altered in all types.

Group B cases.

The total number of cases investigated under this head was 35. From an analysis of the results four types were differentiated (Graph 15). Only the average value in each type has been given.

Interpretation of the results :

- (i) There is a more or less marked fall in the total protein in all types except type I.
- (ii) The albumin fraction is markedly diminished in all types, the lowest figure being obtained in type IV.
- (iii) The globulin fraction is normal in type III, moderately increased in types II and IV and markedly increased in type I.
- (iv) The albumin : globulin ratio is reversed, particularly in types I and IV.

DISCUSSION.

Carbohydrate metabolism.—Our results show that :—

- (i) The blood sugar in the majority of the starvation cases was lower than the average normal.
- (ii) Two distinct types of sugar tolerance curves were obtained from the two groups of cases of starvation.

In group A, the majority of the cases showed defective carbohydrate tolerance and the blood-sugar curves resembled those seen in cases of diabetes of varying degrees of severity. A detailed inquiry into the cause of this variation in the degree of defect in the carbohydrate utilization of these people revealed that this depended mainly on the degree of starvation ; those undergoing short but not acute starvation showed either normal or slightly defective carbohydrate tolerance, whereas those undergoing short and acute starvation gave results resembling those seen in cases of diabetes of moderate or even severe degree.

The group B cases, on the other hand, gave an entirely different type of results. Here the ingestion of even 100 g. of glucose made no impression on the blood-sugar level at all. In a few cases in this group, it was found that the blood sugar, after the glucose meal, showed an even further decrease below the initial level at the end of the second and third hours. The reason for this peculiar behaviour of blood sugar after the glucose meal is not clear though we have obtained similar results in anæmia and sprue.

The intravenous glucose tolerance test, however, suggested that the failure of the blood sugar to rise after the glucose meal was due to impaired absorption, some of them showed normal utilization of glucose. Others, however, showed defective glucose utilization, sometimes to a marked degree.

It is suggested that malnutrition due to starvation may lead to a condition of the alimentary canal where the power of absorption even of glucose may be partially or even completely lost.

It is evident that starvation reduces the blood-sugar level and causes a definite impairment of the glucose-utilization power of the individual. The effect of starvation on the blood-sugar level was studied by Morgulis and Edwards (1924) in animals and Shope (1927) and Lennox, O'Conner and Bellinger (1928) in human subjects. They all found definite lowering of the blood-sugar level in starvation. Reduced ability for glucose utilization in starvation in animals was observed by Lehmann (1874) and Hofmeister (1890). Shope (*loc. cit.*) made similar observations on human subjects. Himsworth (1935) showed that low carbohydrate diet reduces the sugar tolerance.

The reason for lowered carbohydrate tolerance in cases of starvation is not fully understood. Chambers (1938) has suggested that this may be due to defective oxidation in the tissues. Dann and Chambers (1932) have observed that the loss of ability to use glucose is possibly due to the fact that glycconeogenesis has been going on intensively, the newly formed glycogen being at once converted into glucose, the usage of which is reduced. It has been suggested by some workers that the liberation of insulin is depressed. Himsworth (*loc. cit.*), however, is of opinion that sensitivity to insulin is involved rather than the diminution of the pancreatic output.

Serum protein.—The total serum-protein content was found to be low in varying degrees in all cases of starvation. In group A (emaciated cases) the loss of total protein was not so marked except in children, but there was a fair reduction in the albumin fraction and an increase in the globulin fraction. In group B (oedematous cases of chronic malnutrition due to long starvation) the loss of total protein in the majority of the cases was more marked and in some of these extremely low values were obtained. The albumin fraction was markedly low whereas the globulin fraction was high.

The fall in serum protein does not, however, represent a uniform and proportionate decrease of its two constituents (albumin and globulin) and herein lies the importance of our findings. A critical survey of our results clearly shows that the albumin fraction of the protein is more or less markedly decreased in most cases, more particularly in the cases of B group, whereas the globulin fraction was either normal or markedly increased. It is thus evident that the decrease in the total protein content of the blood was due entirely to the diminution of the albumin fraction.

The fact that the albumin fraction of the serum is decreased while the globulin fraction either remains normal or is increased results in a serious alteration of the normal 2:1 albumin:globulin ratio. In some of the group B cases where the albumin fraction was markedly decreased and the globulin fraction markedly increased there was a reversal of the albumin:globulin ratio. Weech (1936) also noted alteration in the blood albumin:globulin ratio to 2:2 instead of the normal 4:2.

Oedema, as has already been stated, was an associated factor in the group B cases, the main reason being the marked decrease in the albumin content of the blood. Oedema among the inhabitants of famine-afflicted areas was studied in Europe during the last Great War. Knack and Neuman (1917), and Bruckman, d'Esopo and Peters (1930) observed a low level of plasma protein due to lowering of the albumin content in persons suffering from famine oedema as well as in malnutrition oedema.

In the majority of group A cases, the globulin content of the blood was within normal limits, the only exception being in type III cases in which it was definitely high. These cases were suffering from complications particularly malaria and dysentery.

Group B cases however show some interesting results. In type I the globulin content was very high in all cases having kala-azar. In type II the globulin content was also high (being about one-and-a-half times the albumin content) and these showed complications of some form or other, such as malaria, dysentery, etc.

Apart from severe oedema and ascites no evidence of any intercurrent disease could be elicited in type IV cases.

Thus, except in the 4 cases of type IV, all the cases having a high globulin content had evidence of some form of intercurrent diseases. Similar observations have also been made by Rowe (1916) and Sia (1921) who observed an increase in the serum globulin in infectious conditions. Bruckman *et al.* (*loc. cit.*) also demonstrated increased serum globulin in cases of malnutrition associated with infection while albumin was extremely low.

Although the division of the cases into groups A and B was originally made on the history and the physical findings on admission (prior to the commencement of the biochemical investigations), the subsequent laboratory findings corroborated the clinical classification. The difference between the two groups, particularly with regard to carbohydrate metabolism and serum-protein content, was so remarkably clear cut that the results of biochemical tests could be predicted.

It has not been possible from our results to correlate any particular type of the sugar-tolerance curve with any definite type of the serum-protein content values.

SUMMARY AND CONCLUSION.

1. Prolonged starvation lowers the blood-sugar level. In some cases figures as low as 0.040 per cent were observed.

2. In certain groups of starvation cases marked lowering of carbohydrate tolerance was observed while in other groups absorption of glucose from the alimentary tract was found to be well below normal.

3. The total serum-protein content of the blood in starvation cases was found to be universally low. This affected the albumin fraction only. In cases with marked oedema the fall in the albumin (as also in the total protein) was sometimes of an extreme degree.

4. The globulin content of the blood was not decreased but was normal or increased. This was particularly obvious in cases with concurrent infection as in the oedema cases.

5. The albumin: globulin ratio was markedly altered. Sometimes, in extreme cases, a reversal of the normal ratio was obtained.

6. Non-protein nitrogen and uric acid were high in the majority of cases. The phospho-lipoid content was found to be exceptionally high in some cases. The cholesterol and the calcium content was found to be low in the majority of the cases.

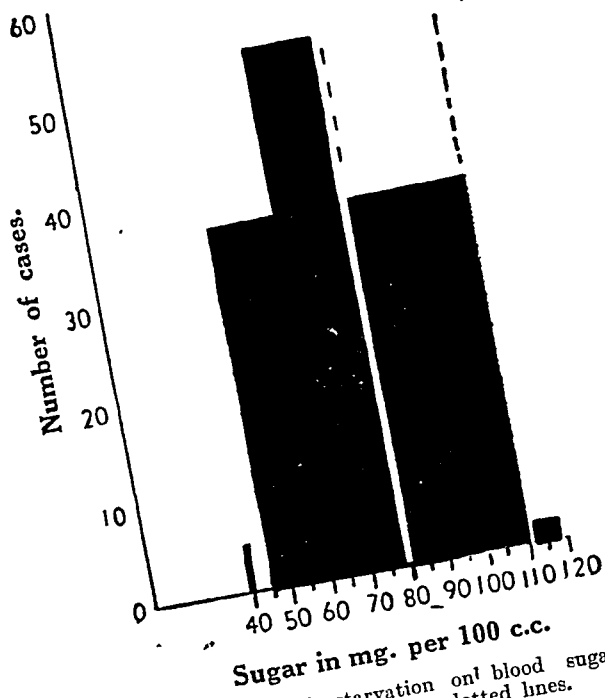
7. Chloride and urea excretion in the urine was low in almost all cases.

The authors take this opportunity of thanking the other workers of the laboratory more particularly Mr. P. B. Sen, Mr. P. K. Pal and Mr. S. K. Ghosh for the keen co-operation and help in carrying out this work.

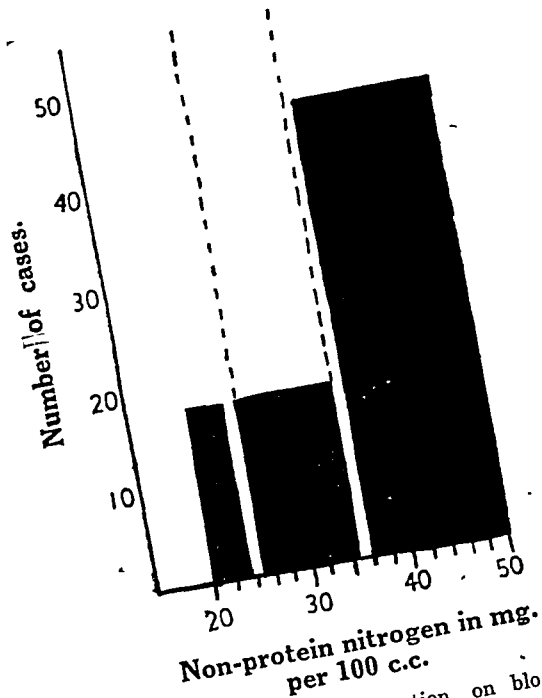
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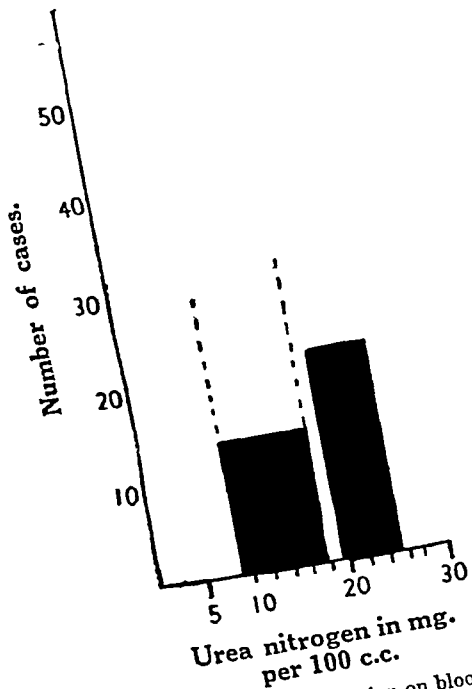
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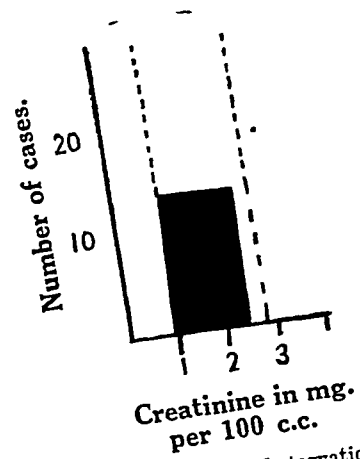
GRAPH 1.—Effect of starvation on blood sugar. Normal range is indicated by dotted lines.



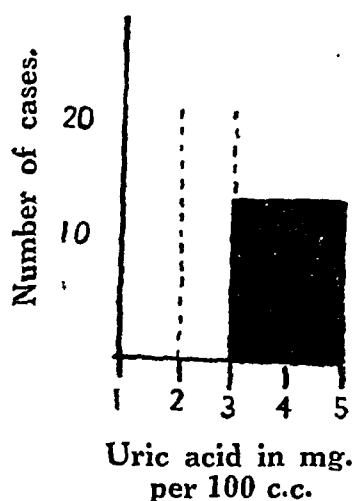
GRAPH 2.—Effect of starvation on blood non-protein nitrogen. Normal range is indicated by dotted lines.



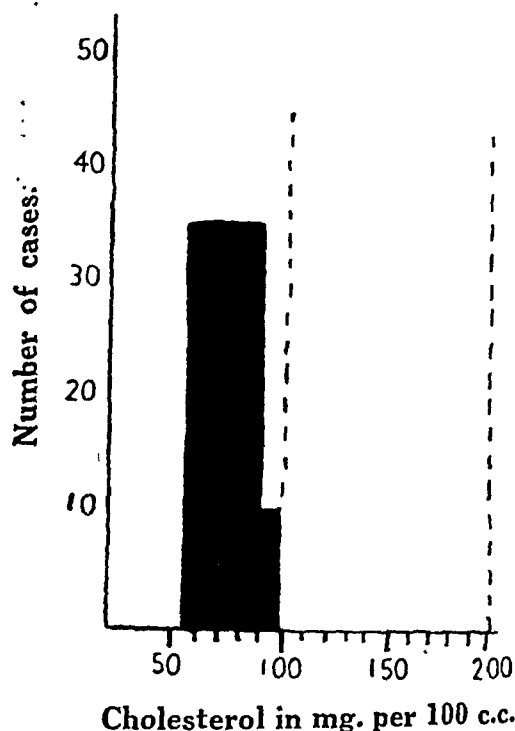
GRAPH 3.—Effect of starvation on blood-urea nitrogen. Normal range is indicated by dotted lines.



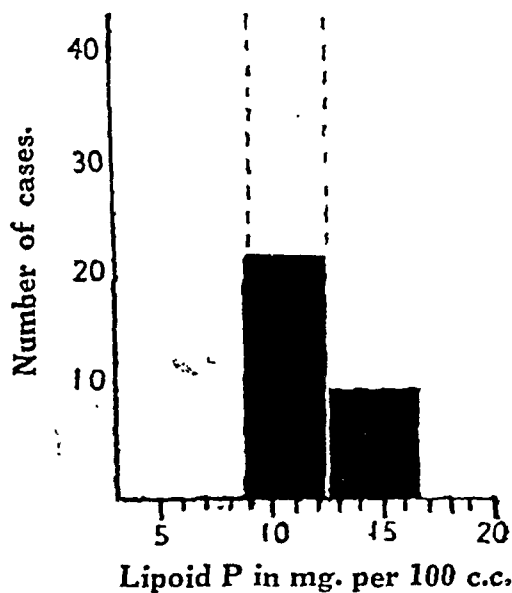
GRAPH 4.—Effect of starvation on blood creatinine. Normal range is indicated by dotted lines.



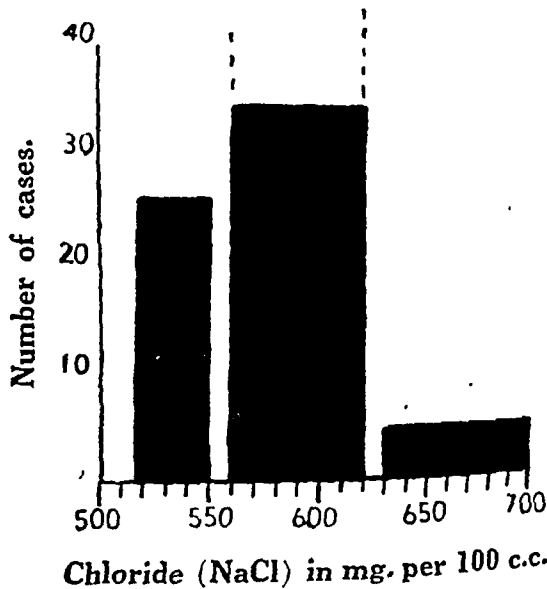
GRAPH 5.—Effect of starvation on blood-uric acid. Normal range is indicated by dotted lines.



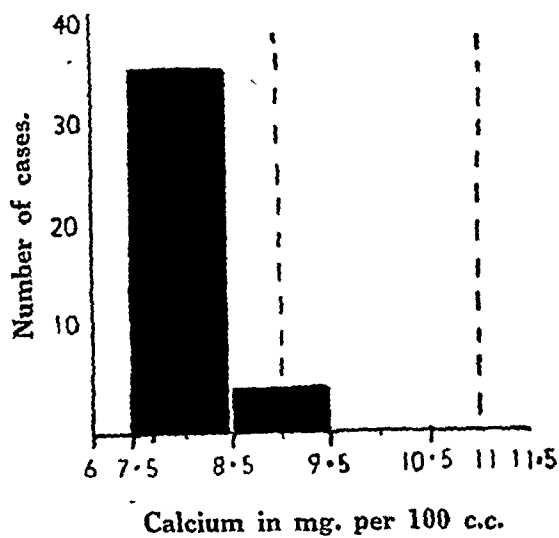
GRAPH 6.—Effect of starvation on blood cholesterol. Normal range is indicated by dotted lines.



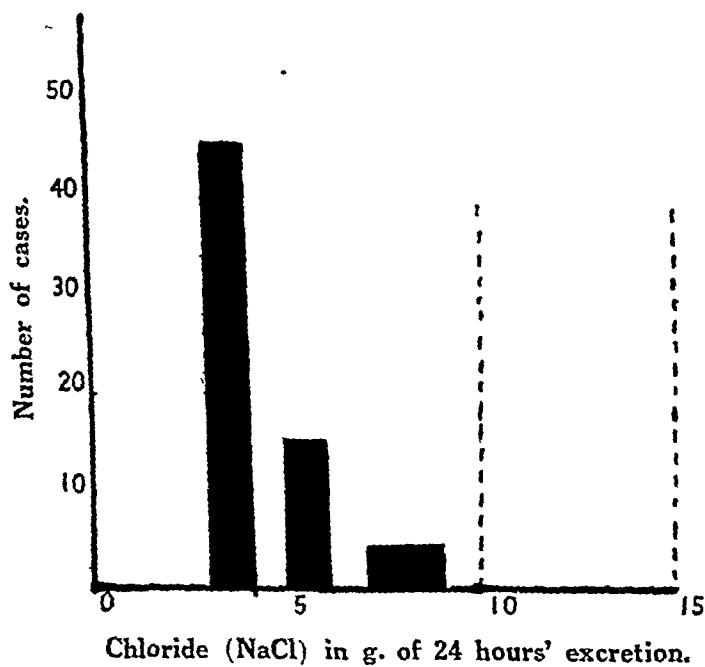
GRAPH 7.—Effect of starvation on blood-lipoid P. Normal range is indicated by dotted lines.



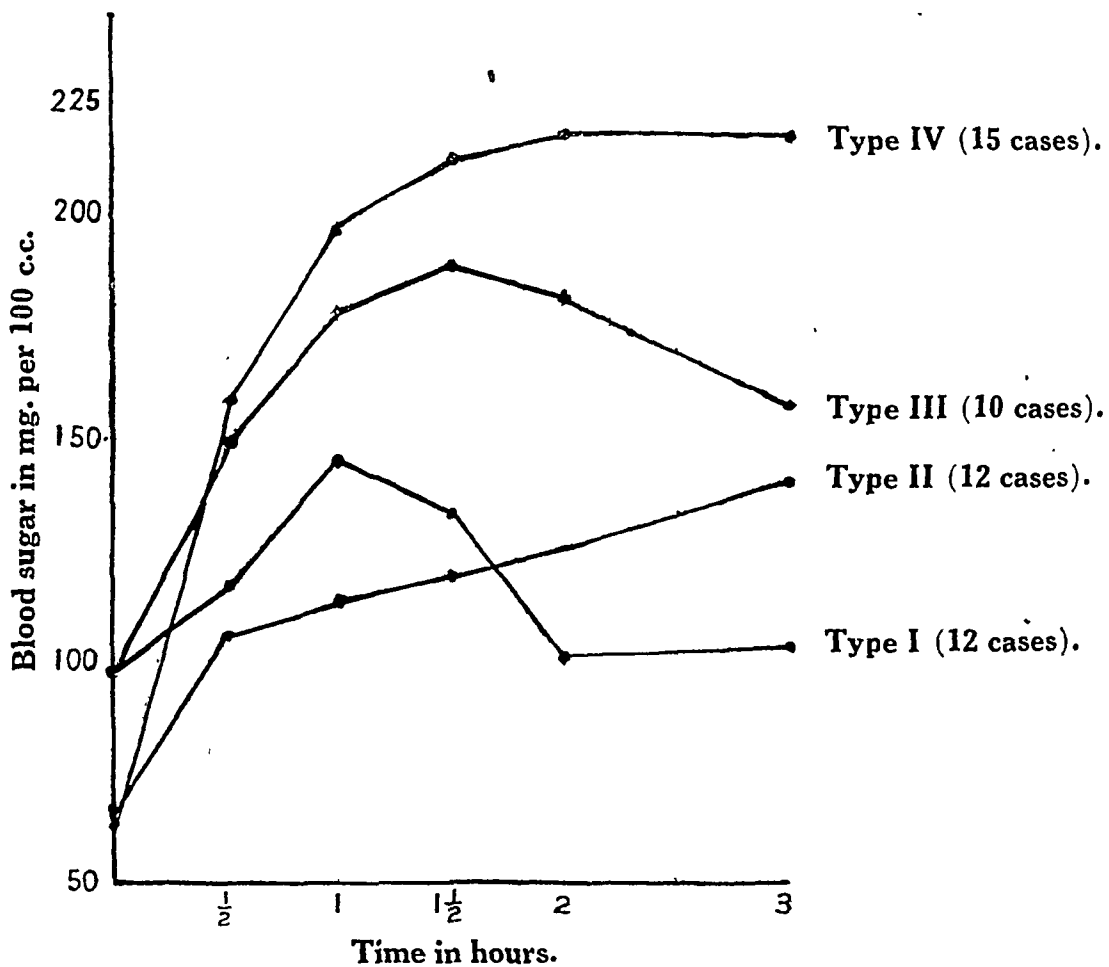
GRAPH 8.—Effect of starvation on plasma chloride. Normal range is indicated by dotted lines.



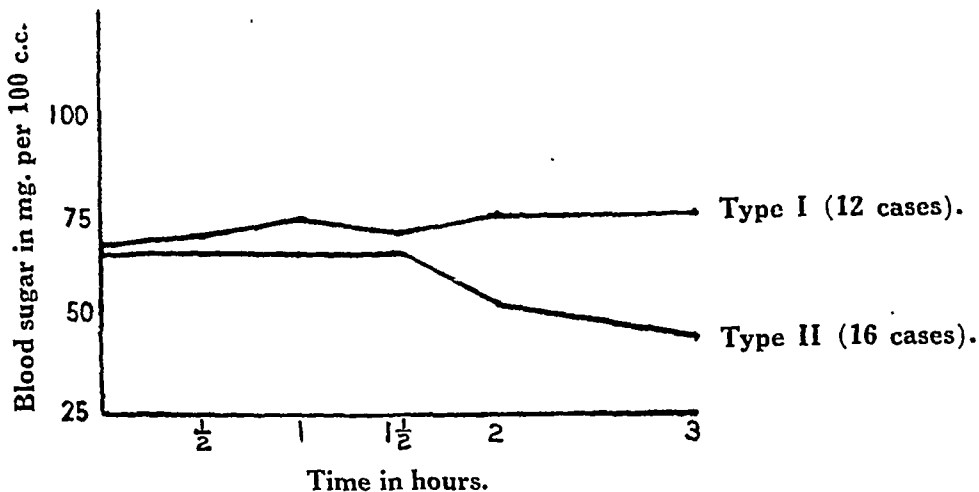
GRAPH 9.—Effect of starvation on blood calcium.
Normal range is indicated by dotted lines.



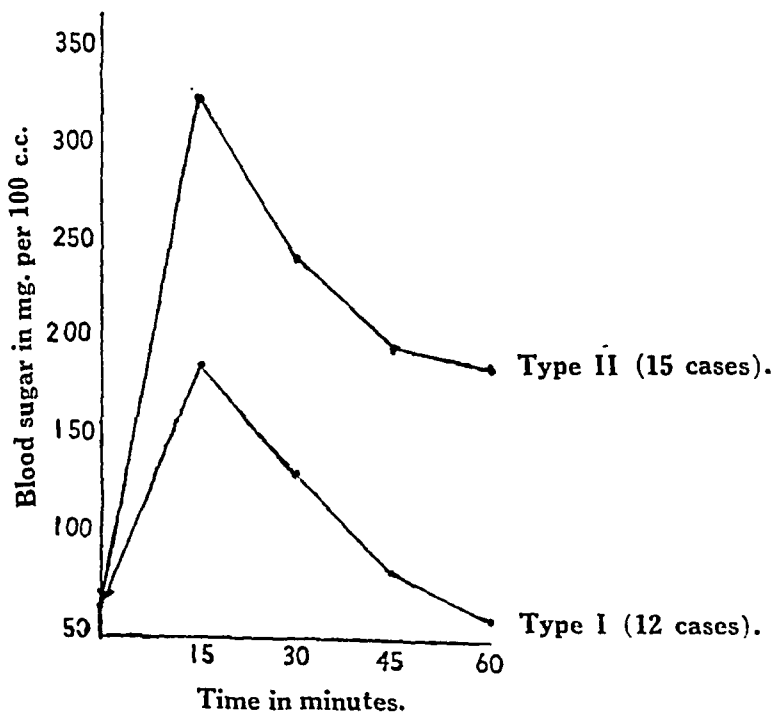
GRAPH 10.—Effect of starvation on chloride excretion. Normal range is indicated by dotted lines.



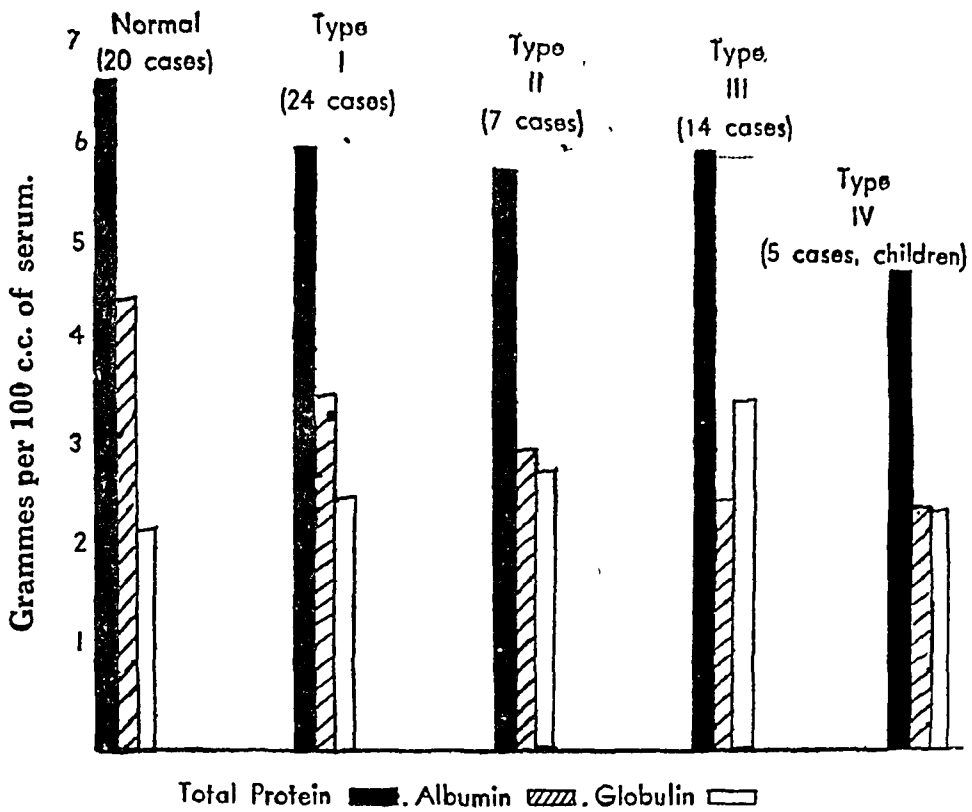
GRAPH 11.—Effect of oral administration of 50 g. of glucose on blood sugar in starvation group A cases.



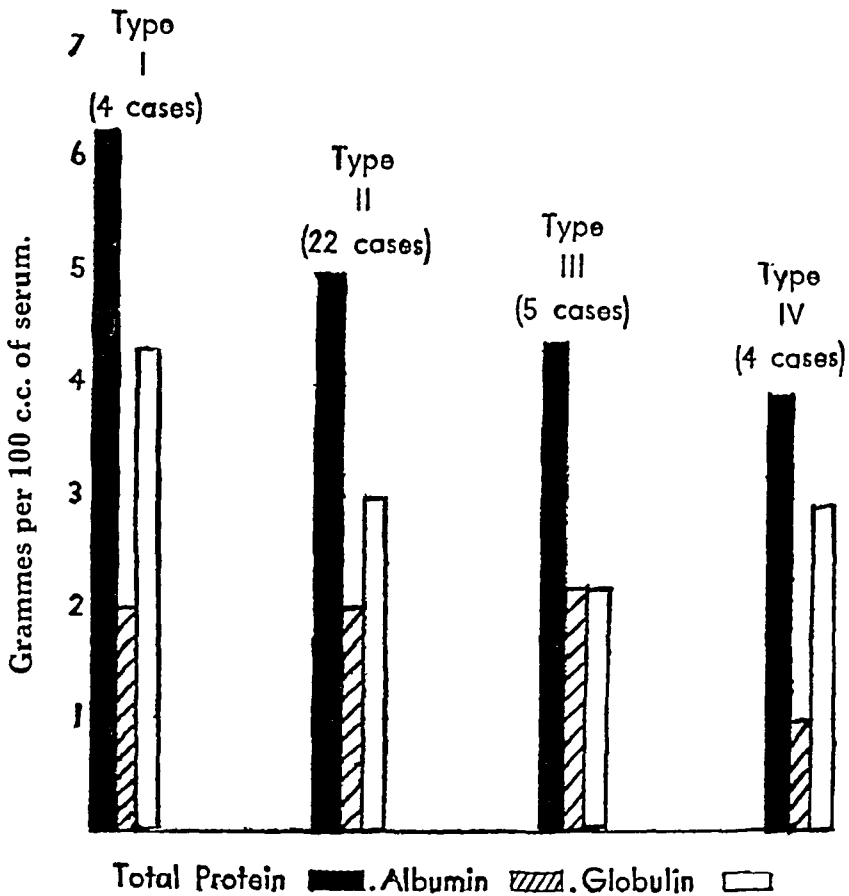
GRAPH 12.—Effect of oral administration of 50 g. of glucose on blood sugar in starvation group B cases.



GRAPH 13.—Effect of intravenous administration of glucose (0.2 g. per kilo) on blood sugar in starvation group B cases.



GRAPH 14.—Effect of starvation on serum proteins in group A cases.



GRAPH 15.—Effect of starvation on serum proteins in group B cases.

STUDIES ON THE BIOCHEMISTRY OF THE 'CHOLERA-RED' REACTION.

Part I.

BY

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AMINO-ACIDS in culture media undergo various types of breakdown as the result of bacterial metabolism. These breakdown processes are due chiefly to de-amination or decarboxylation or both. Various diagnostic tests in bacteriology are based on the presence or absence of a particular end-product in the culture medium after bacterial growth. The production of 'cholera-red' reaction in the culture medium is one such test used for the identification of *V. cholerae*. It is now known that this reaction is not specific for *V. cholerae* as other vibrios also give this reaction. The absence of any positive reaction is, however, an important finding. This reaction is due to the presence of a nitroso derivative either of indole or of a compound containing indole ring derived from tryptophan and nitrate present originally in the culture medium (Hewlett, 1901). It is, however, common experience that a positive reaction is not always obtained with *V. cholerae* even when the medium is known to contain tryptophan and a nitrate. Up till now not much is known about the 'cholera-red' reaction except that the presence of some carbohydrate inhibits the reaction. Still less is known about the mechanism of inhibition by various reagents that might be present in culture media and in what respect a medium giving a positive reaction differs from another, which fails to give a positive reaction.

The inhibitory effect of glucose may be due either to its reducing property, whereby the course of breakdown of tryptophan is diverted from the direction necessary for positive reaction or to a complex formation between tryptophan and glucose (Homer, 1916). This complex may not be easily attacked by the organism and thereby tryptophan may be spared from breakdown. The influence of glucose in inducing an anaerobic or reducing environment in a culture medium is well known. If this particular property of glucose be the cause of negative 'cholera-red' reaction, then it is natural to expect that other reducing agents, which are common in any meat extract or peptone, will also play a similar rôle. Oxidizing agents should overcome this inhibition. The present work was undertaken to study the above aspects experimentally and to investigate the chemistry of the 'cholera-red' reaction more fully than has hitherto been done.

EXPERIMENTAL.

One per cent bacto-peptone solutions containing 0.5 per cent sodium chloride and different amounts of glucose were prepared. The minimum amount of glucose that inhibits positive 'cholera-red' reaction in 18-hour culture was determined. Separately, the maximum amount of a few oxidizing and reducing agents that will not stop the reaction in 1 per cent bacto-peptone medium were determined. The important reducing agents present in meat extract or in any commercial peptone include cysteine and glutathione (reduced). As these are capable of reversible oxidation-reduction change, their concentration is an important feature in determining the environment which the medium provides for the growth of bacteria. Cysteine is taken as representing this type of agent. These oxidizing and reducing agents were added in different amounts to the bacto-peptone solution

and the 'cholera-red' reaction was tested after 18-hour growth of *V. cholerae*. Results are recorded in Table I:—

TABLE I.

Reagents.				Concentration of various reagents in mg. per 100 c.c.							
				10	5	2.5	1.5	1.25	1.0	0.5	0.25
Glucose	±	+	+	+	+	+	+	+
Sodium nitrate, NaNO_3	—	—	—	—	—	—	+	+
Copper sulphate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	—	+	+	+	+	+	+	+
Potassium ferricyanide, $\text{K}_3\text{Fe}(\text{CN})_6$	—	—	—	+	+	+	+	+
Potassium ferrocyanide, $\text{K}_4\text{Fe}(\text{CN})_6$	—	—	—	+	+	+	+	+
Cysteine	—	+	+	+	+	+	+	+

+ signifies positive reaction and — negative reaction in this and all subsequent experiments. Maximum amounts of glucose and nitrate that fail to inhibit the reaction have been found to vary slightly with different lots of peptone, probably owing to variation in the amounts of oxidizing and reducing agents present.

In order to ascertain whether the other reagents influence the permissible limit of glucose for positive reaction, the maximum amount of these, which were not inhibitory to positive reaction, were separately added to peptone water containing different amounts of glucose. Reactions observed are recorded in Table II:—

TABLE II.

Glucose.				Concentration of reagents in mg. per 100 c.c.				
				NaNO_3 0.5	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 5.0	$\text{K}_3\text{Fe}(\text{CN})_6$ 1.5	$\text{K}_4\text{Fe}(\text{CN})_6$ 1.5	Cysteine. 5.0
5	+	+	+	+	+
10	+	+	+	—	—
15	+	+	+	—	—
18	+	+	+	—	—
Molar conc.	$100 \times 10^{-5} \text{M}$...	$3 \times 10^{-5} \text{M}$	$20 \times 10^{-5} \text{M}$	$5 \times 10^{-5} \text{M}$	$5 \times 10^{-5} \text{M}$	$4 \times 10^{-5} \text{M}$	
20	+	+	—	—	—
25	±	±	—	—	—

± signifies slight reaction.

To determine whether the concentrations of the salts have any ionic effect on the reaction, the maximum amount of glucose (20 mg. per 100 c.c.) was added to peptone water

containing different amounts of the various salts up to the maximum permissible limit (*vide* Table II). The results are noted in Table III :—

TABLE III.

Medium : One per cent bacto-peptone solution containing 20 mg. glucose per 100 c.c.

Reagents.	Concentration in mg. per 100 c.c.					
	5	2.5	1.5	1.0	0.5	0.25
Sodium nitrate, NaNO_3 ...	—	—	—	—	+	+
Copper sulphate, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$.	+	+	+	+	+	+
Potassium ferricyanide, $\text{K}_3\text{Fe}(\text{CN})_6$.	—	—	+	+	+	+
Potassium ferrocyanide, $\text{K}_4\text{Fe}(\text{CN})_6$.	—	—	—	—	—	—

The results indicate that within limits, variations in the ionic concentration have no appreciable effect on the reaction.

From Table II it appears that addition of different amounts of reducing agents to the media lowers the permissible limit of glucose for a positive reaction. If oxidizing agents can raise the limit of glucose to the original level, the effect of a reducing environment in stopping the reaction will be evident. The effect of adding different oxidizing and reducing agents with glucose on the 'cholera-red' reaction are given in Table IV. The concentration of each reagent is the maximum that individually gives a positive reaction in bacto-peptone.

TABLE IV.

Concentration of reagents in mg. per 100 c.c. bacto-peptone solution.

Glucose.	1.5 ferrocyanide plus			5.0 cysteine plus		
	$\text{K}_3\text{Fe}(\text{CN})_6$, 1.5	NaNO_3 , 0.25	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5.0	$\text{K}_3\text{Fe}(\text{CN})_6$, 1.5	NaNO_3 , 0.25	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 5.0
5	+	+	+	+	+	+
10	+	+	+	+	+	+
15	—	+	+	±	+	+
20	—	+	+	—	+	+

It appears from Table IV that the effect of ferrieyanide is not so pronounced as that induced by nitrate and copper sulphate.

It was thought desirable to study whether the presence of free tryptophan is necessary and whether tryptophan in combination would give the reaction. Witte's peptone was freed from amino-acids including tryptophan by saturating a 10 per cent solution of it with ammonium sulphate. The precipitate (proteose fraction) was freed from ammonium sulphate by dialysis. Taking bacto-peptone to contain 16 per cent nitrogen, solutions were prepared of (a) Witte's peptone, (b) fraction of Witte's peptone precipitated by ammonium sulphate and (c) bacto-peptone, all containing 0.16 per cent nitrogen. The amount of tryptophan necessary to give a positive reaction, was determined by adding different amounts of tryptophan to casein-hydrolysate solution (0.16 per cent N) containing 0.25 mg. of NaNO_3 per 100 c.c. to supply the requisite amount of nitrate. The hydrolysate was prepared by boiling a 10 per cent suspension of casein in 20 per cent HCl for 20 hours at 125°C . Excess hydrochloric acid was removed in vacuum and the solution was clarified with pure animal charcoal. Boiling a protein with mineral acid destroys all tryptophan present in it and the hydrolysate is taken to be free of tryptophan. The bromine test for free tryptophan was carried out in each case. Results are given in Table V:—

TABLE V.

	Bacto-peptone.	Witte's peptone.	Proteose fraction.	Casein hydrolysate containing added tryptophan, mg. per 100 c.c.				
				0	1	2	3	6
Bromine test for free tryptophan	...	+	—	—	+	+	+	+
'Cholera-red' reaction	...	+	+	—	—	—	+	+

It appears that the presence of free tryptophan is not essential for the reaction. A positive reaction is also obtained when tryptophan is in a combined state. In the case of free tryptophan, the minimum amount necessary was found to be about 3 mg. per 100 c.c.

DISCUSSION.

The fact that glucose, cysteine and ferrocyanide exert an inhibitory influence on the 'cholera-red' reaction (Table II) suggests that their reducing effect is responsible for the negative result. The beneficial effect obtained by the oxidizing agents in promoting the reaction gives additional support to this view (*vide* Tables II and IV). Colour reactions similar to 'cholera-red' have been obtained by Woods (1935) with pure indole acetic and indole by addition of concentrated H_2SO_4 and NaNO_2 to the solution of each. This has been confirmed by us. All these go to show that the reducing agents present in the culture medium lead to the production of indole propionic acid from tryptophan and this fails to give the specific test for 'cholera-red'.

As culture in bacto-peptone solution which contains no added glucose gives a positive reaction, it appears reasonable to suppose that the oxidative breakdown of tryptophan will follow automatically in a medium containing glucose, if only the reducing effect of the latter is counter-balanced. The oxidation-reduction nature of any system does not depend on the total quantity of the respective reagents present but is dependent on the ratio of their amounts. If the system is capable of reversible transformation, the smallest amount of the reagents should produce the reaction. As will be seen from Table III, addition of minute amounts of oxidizing agents brings out a positive test which is otherwise negative. In presence of molecular oxygen, these oxidizing substances are capable of reversible reduction and oxidation and hence a minute amount of these will be able to induce an oxidizing potential. The fact that the effects of various agents are not found within the permissible

limit, to depend on the ionic concentration of the salts (*vide* Table III), goes to establish that the oxidative effect is 'catalyst' in nature. Homer (*loc. cit.*) working with *B. coli* suggested that the failure of indole production from tryptophan in presence of glucose is due to a complex formation between tryptophan and glucose and that this complex is not easily attacked by bacteria. Theoretically, two moles of the oxidizing agents used here should be required for one mole of glucose if it be a chemical combination. But the amount of different oxidizing agents actually found necessary to give a positive 'cholera-red' reaction (*vide* Table II) are too small to warrant this assumption of chemical combination.

Glucose is oxidized irreversibly and hence does not influence the oxidation-reduction potential of the substrate to any appreciable extent, when another reversible system is present. Yet there is a limit to the amount of glucose that can be added (*vide* Table II). The 'redox' potential of a system is primarily a function of hydrogen-ion concentration and as the product of glucose oxidation is an acid, it sets a limit to the amount of glucose that may be allowed in the substrate.

SUMMARY.

1. The inhibitory effect of glucose on the positive 'cholera-red' reaction in a broth culture has been shown to be due to its reducing influence. Other reducing agents are capable of playing the same rôle.

2. The theory of glucose-tryptophan complex as explaining the failure to obtain a positive 'cholera-red' reaction appears untenable on the basis of experimental observation.

3. The addition of minute amounts of oxidizing agents, capable of undergoing reversible change, can catalytically overcome the influence of reducing agents.

My thanks are due to Dr. Phanindra Nath Basu, M.B., and Mr. Diptish Chandra Chakraborty, B.Pharm., for useful suggestions and technical assistance.

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'Cholera-red' reaction	...	+	+	+	—	—	—	+	+

It appears that the presence of free tryptophan is not essential for the reaction. A positive reaction is also obtained when tryptophan is in a combined state. In the case of free tryptophan, the minimum amount necessary was found to be about 3 mg. per 100 c.c.

DISCUSSION.

The fact that glucose, cysteine and ferrocyanide exert an inhibitory influence on the 'cholera-red' reaction (Table II) suggests that their reducing effect is responsible for the negative result. The beneficial effect obtained by the oxidizing agents in promoting the reaction gives additional support to this view (*vide* Tables II and IV). Colour reactions similar to 'cholera-red' have been obtained by Woods (1935) with pure indole acetic and indole by addition of concentrated H_2SO_4 and NaNO_2 to the solution of each. This has been confirmed by us. All these go to show that the reducing agents present in the culture medium lead to the production of indole propionic acid from tryptophan and this fails to give the specific test for 'cholera-red'.

As culture in bacto-peptone solution which contains no added glucose gives a positive reaction, it appears reasonable to suppose that the oxidative breakdown of tryptophan will follow automatically in a medium containing glucose, if only the reducing effect of the latter is counter-balanced. The oxidation-reduction nature of any system does not depend on the total quantity of the respective reagents present but is dependent on the ratio of their amounts. If the system is capable of reversible transformation, the smallest amount of the reagents should produce the reaction. As will be seen from Table III, addition of minute amounts of oxidizing agents brings out a positive test which is otherwise negative. In presence of molecular oxygen, these oxidizing substances are capable of reversible reduction and oxidation and hence a minute amount of these will be able to induce an oxidizing potential. The fact that the effects of various agents are not found within the permissible

STUDIES ON THE BIOCHEMISTRY OF THE 'CHOLERA-RED' REACTION.

Part II.

BY

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VARIOUS reducing agents have been found (Sen, 1946—Part I of this series, see p. 151) to inhibit 'cholera-red' reaction in a culture of *V. cholerae*. They act either by inhibiting the formation of the enzyme concerned or by diverting the course of enzymic reaction so that the particular end product to which positive reaction is due is not formed. Following work was undertaken with a view to elucidate these possibilities and to study further the nature of the enzyme concerned.

EXPERIMENTAL.

A smooth strain of *V. cholerae* was grown in one per cent bacto-peptone solution containing 20 mg. glucose per 100 c.c. After 18-hour growth, part of the culture on test gave a negative 'cholera-red' reaction (cf. Table I. Part I, p. 152). The rest of the culture was centrifuged and the supernatant passed through a Seitz filter pad (E. K. 14) and the filtrate collected. The centrifuged bacterial deposit was washed thrice and finally re-suspended in normal saline. Portions of this cell suspension were then added to solutions containing tryptophan and sodium nitrate with and without glucose and the substrates were tested after incubation at 37°C. for periods of 30, 60 and 90 minutes. The final strength of the bacterial suspension in this and all subsequent experiments was 4,000 million organisms per c.c. Similar tests were done with the bacteria-free filtrates. Results are recorded in Table I:—

TABLE I.

Substrate.	Period of incubation before test.		
	30 min.	60 min.	90 min.
1. Bacterial suspension + test solution. (Final strength: tryptophan 6 mg. and sodium nitrate 0.25 mg. per 100 c.c. Bacterial cells 4,000 millions per c.c.)	+++	++++	+++++
2. Cell-free filtrate 4 c.c. + test solution. (Final strength of tryptophan and sodium nitrate as in No. 1.)	±	+	++

+ indicates positive reaction, which increased with time.

Though the filtrate showed definite enzymatic activity increasing progressively with time, the intensity of the reaction was slight in comparison with that obtained with the cells. Hence for convenience subsequent work was done with washed vibrios in the 'resting phase'.

As breakdown of tryptophan is involved in this reaction, it was considered to be of interest to compare the enzymatic activity of cells grown in medium containing tryptophan with those grown in medium free from this amino-acid.

V. cholerae was grown on agar slopes containing (a) 1 per cent bacto-peptone, (b) 1 per cent Witte's peptone, and (c) casein hydrolysed by hydrochloric acid (*vide* Part I). This hydrolysate was so diluted as to contain nitrogen equivalent to 1 per cent bacto-peptone solution in the final medium. Portions from the three cell suspensions (washed 18-hour growth) were heated at 56°C. for 30 minutes, so as to kill the organisms. The three living and the three killed suspensions were added to different tubes containing (a) tryptophan and sodium-nitrate solution, (b) one per cent bacto-peptone solution, and (c) one per cent Witte's peptone solution. Reactions noted on addition of concentrated sulphuric acid after one hour are recorded in Table II :—

TABLE II.

Solid medium used for growing the vibrio.	FINAL CONCENTRATION OF THE INGREDIENTS IN THE SUBSTRATES.		
	Tryptophan 6 mg. + NaNO ₃ 0.25 mg. per 100 c.c.	One per cent bacto-peptone.	One per cent Witte's peptone.
1. Bacto-peptone agar	++	+	—
2. „ (bacterial suspension heated)	—	—	—
3. Witte's peptone agar	++	+	—
4. „ (bacterial suspension heated)	—	—	—
5. Casein hydrolysate agar	—	—	—
6. „ (bacterial suspension heated)	—	—	—

As glucose and other reducing agents present in culture media affect the reaction (*vide* Part I), it may be expected that similar inhibitory effect will also be observed with washed cells in presence of glucose. Oxidizing agents should overcome this effect if it be due to its reducing property. Sodium nitrate and copper sulphate were added to cell suspensions in tryptophan solution and in bacto-peptone solution. Different amounts of glucose were also added. Tests for 'cholera-red' reaction were made after incubation at 37°C. for one hour. Results obtained are recorded in Table III :—

TABLE III.

SUBSTRATES.									
Cell suspension + tryptophan 6 mg. per 100 c.c. + NaNO ₃ 0.25 mg. per 100 c.c.				Cell suspension + 2 per cent bacto-peptone solution + tryptophan * 2 mg. per 100 c.c. + NaNO ₃ 0.25 mg. per 100 c.c. Final strength of bacto-peptone comes to one per cent.					
Glucose added (mg. per 100 c.c. of final mixture) :—	10	20	40	60	10	20	40	60	
1. Control	++	+	±	—	±	—	—	—	—
2. NaNO ₃ (0.25 mg. per 100 c.c.)	++	+	+	±	++	+	±	—	—
3. CuSO ₄ .5H ₂ O (1 mg. per 100 c.c.)	++	+	+	±	+	+	±	—	—
4. CuSO ₄ .5H ₂ O (5 mg. per 100 c.c.)	±	—	—	—	++	+	+	—	—

* Tryptophan was added to increase the intensity of colour.

The addition of sodium nitrate shows a beneficial result. The addition of copper sulphate in concentration used in previous experiments (Part I) makes the reaction negative. In a parallel experiment with cells suspended in one per cent bacto-peptone solution with copper sulphate and with sodium nitrate definite enhancement of the reaction was obtained (Table III). The presence of a strong oxidizing environment as induced by higher concentrations of copper sulphate (No. 4, Table III) appears to be unfavourable in the case of washed cells suspended in tryptophan solution. Bacto-peptone, probably by virtue of the reducing agents it contains, balances the oxidizing effect of copper sulphate (Table III).

As the reduction of nitrate to nitrite is essential for a positive reaction, tests for nitrite were done (Shinn, 1941) in different substrates containing cell suspension and nitrate. Results obtained after one hour's incubation at 37°C. are recorded in Table IV:—

TABLE IV.

Substrate.							Test for nitrite.
1. Cell suspension + sodium nitrate	—
2. Cell suspension + sodium nitrate + glucose	+
3. Cell suspension + sodium nitrate + copper sulphate (5 mg. per 100 c.c.)	—
4. Cell suspension + sodium nitrate + glucose + copper sulphate (5 mg. per 100 c.c.)	—
5. Cell suspension + sodium nitrate + glucose + copper sulphate (2 mg. per 100 c.c.)	±
6. Cell suspension + sodium nitrate + glucose + copper sulphate (1 mg. per 100 c.c.)	+

Final concentration of sodium nitrate—0.25 mg. in 100 c.c.

Final concentration of glucose—20 mg. in 100 c.c.

In No. 1 reduction of nitrate does not occur as there is no substance to act as an electron donor (Ingold, 1934). With the addition of glucose, reduction of nitrate to nitrite takes place in No. 2. In experiment No. 5, with a smaller amount of copper sulphate a faint positive reaction for nitrite is obtained, while in experiment No. 6 with still less copper sulphate the amount of nitrite formed was practically equal to that in No. 2. In cases of Nos. 3 and 4, nitrite is not formed. The strong oxidizing effect of copper sulphate most probably interferes with the reduction of nitrate to nitrite. This reduction is essential for a positive 'cholera-red' reaction. Hence the failure to get a positive test, when copper sulphate (5 mg. per 100 c.c.) was added to the emulsion containing tryptophan and nitrate (expt. No. 3, Table III).

DISCUSSION.

When the presence of an enzyme in the cell is spoken of, it is meant that the cells manifest certain specific activity, which identifies the enzyme. It may not be always possible to separate the enzyme from the cell. In this particular case, though the culture filtrate shows definite enzymic activity increasing with time (Table I) the weak intensity of the reaction shows that the enzyme is not separable from the cells to any great extent.

A strong positive reaction was obtained with washed vibrios from a culture which failed to give the reaction (Table I). As the positive reaction was obtained within the resting phase of the cells, it appears that the initial negative reaction with whole culture was due to non-formation of the specific end product, though the enzyme was present.

It will be observed from Table II that within the time of experiment (resting phase of the vibrio) the presence of free tryptophan is necessary for a positive reaction. Cells

suspended in Witte's peptone solution which contains no free tryptophan (*cf.* Part I) failed to give a positive reaction in all cases. Liquid culture of *V. cholerae* in Witte's peptone solution, however, gives a positive reaction. It appears that in cultures in Witte's peptone solution tryptophan is at first liberated from the combined state and subsequently acted upon by the specific enzyme. The unheated cells from Witte's peptone and bacto-peptone media, when suspended in bacto-peptone solution which contains free tryptophan, gave positive reaction. Cells grown in medium prepared with acid hydrolysate of casein in which tryptophan is destroyed failed to show enzymic activity in all cases. This particular enzyme, as judged by the 'cholera-red' reaction, therefore, belongs to the group of 'adaptive' enzyme. It only develops in presence of tryptophan in culture media. It also appears that the enzyme is destroyed if heated at 56°C. for 30 minutes.

Happold and Hoyle (1936) working with washed cells of *B. coli* grown on a medium free from tryptophan observed that tryptophan can be broken to indole by cells grown on tryptophan-free medium. Fildes (1938), however, observed that when grown in presence of tryptophan cells of *B. coli* exhibit marked enhancement of this tryptophan-splitting activity. With *V. cholerae*, we have not been able to detect the breakdown of tryptophan by the cells grown on tryptophan-free medium at least within the short period of the experiment (resting phase of the vibrio). Duration of the experiment carried out by Happold and Hoyle was, however, much longer than that of ours. We have observed breakdown of tryptophan within a period as short as 30 minutes (*vide* Table I) by cells grown on media containing tryptophan.

Happold and Hoyle (*loc. cit.*) and Evans *et al.* (1941) suggested that this failure to liberate indole from tryptophan by *B. coli* in presence of glucose, was due to non-liberation of the specific enzyme. With washed cells of *V. cholerae* (Table I) taking the 'cholera-red' reaction to be an indication of breakdown of tryptophan, we could not corroborate this view. That the inhibitory influence of glucose is overcome by oxidizing agents (*vide* Table III) and a positive reaction is obtained, strongly suggests that the inhibition by glucose is due to its reducing action.

Incidentally, it has been observed by us that the technique of 'cholera-red' reaction can be conveniently modified. Culture of *V. cholerae* is made on any common solid medium; after incubation period of about 5 to 6 hours an emulsion is made and added to sterile stock solutions of tryptophan and sodium nitrate. On addition of sulphuric acid after 45 to 60 minutes, the cherry-red colour of 'cholera-red' reaction is obtained. This technique is fairly rapid and avoids the use of any selective medium.

SUMMARY.

1. Liberation of specific enzyme for breakdown of tryptophan takes place in the presence of glucose. But the formation of the specific end product, which gives positive 'cholera-red' reaction is prevented in a reducing environment.

2. The enzyme is separable from the cells to a very small extent. It belongs to the class of adaptive enzymes and is heat labile being destroyed on being heated at 56°C. for 30 minutes.

3. A modified technique for the 'cholera-red' reaction has been suggested.

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STUDIES ON CARBOHYDRATE METABOLISM.

BY

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INTRODUCTION.

NUMEROUS investigations have been carried out by different workers on the glucose content of blood in normal and pathological conditions and its interrelationship with some of the important blood constituents. The administration of glucose is reported to affect the values of blood glucose, potassium and inorganic phosphate contents of serum (Flock *et al.*, 1938).

It has been definitely established that the liver plays an important rôle in the metabolism of carbohydrates (Graham, 1938; Himsworth, 1939) and that van den Bergh's reaction for serum bilirubin is one of the tests for the investigation of hepatic efficiency (Mann and Bollmann, 1935). Somogyi (1942) reported that abnormal diastase levels are indicative of impaired hepatic function. Myers and Killian (1917) had claimed that the diastase activity of blood was increased in diabetes.

In spite of these extensive studies there is no reference in the available literature to a statistical study of the problem in which sufficient data of a comparable nature have been reported whereby the correlation, if any, between these various factors can be determined in normal and pathological subjects. In the present investigation an attempt has been made to interpret statistically the data obtained on the interrelationship of glucose and some of the more important constituents of blood influencing carbohydrate metabolism. Serum inorganic phosphorus and serum potassium values were determined to ascertain the variation in these constituents with blood glucose, before and after ingestion of standard test-dose of dextrose, both in normal and in diabetic subjects. Assays of diastase and bilirubin content of serum and the ascorbic acid content of whole blood were also carried out. The diastatic activity of blood and the bilirubin content of plasma or serum are tests of efficiency of functions which are in some way related to carbohydrate metabolism (Graham, *loc. cit.*; Himsworth, *loc. cit.*; Somogyi, 1942).

This paper presents the results of the above investigations on 94 diabetic subjects (84 males and 10 females)—patients from the various government hospitals in Madras. They were between the ages of 16 and 62 years (average 36.3 years) and the duration of their illness varied from 0.1 to 20 years (average 1.5 years). Control experiments were done on 53 volunteers (31 males and 22 females) from the students and staff of the Medical College, Madras. All these persons were free from diabetes, and their ages varied from 17 to 40 years (average 23.8 years). It is, however, to be noted that since among diabetic patients there is an overwhelming preponderance of males (84 males and 10 females) the differences between males and females on diabetic subjects should be interpreted with caution.

Material and methods.

The subjects were examined early in the morning. Samples of venous blood were collected before, and one hour and two hours after, administration of 50 g. of pure dextrose. Oxalated blood was used for estimation of blood glucose and ascorbic acid while serum was

* Part of the thesis 'Carbohydrate Metabolism—A study of certain blood constituents in diabetes' which formed the basis of the award of the Degree of Master of Science in the University of Madras in 1944, and published by kind permission of the University.

used for the determinations of potassium, inorganic phosphorus, diastase and bilirubin. All the estimations were done in duplicate and the average values were recorded.

Urine was collected immediately after each blood specimen and samples were tested, for presence of glucose, with Benedict's qualitative sugar reagent.

Quantitative determinations of blood constituents were made as follows :—

(1) *Blood glucose*.—This was estimated titrimetrically by the micro-technique of Somogyi-Shaffer-Hartmann (Somogyi, 1930).

(2) *Inorganic phosphate*.—This was estimated by the method of Youngburg and Youngburg (1930). The table of values published by Bodansky (Hawk and Bergeim, 1938, p. 462) was used for calculation.

(3) *Potassium*.—Potassium content of serum was determined colorimetrically by the method of Breh and Gæbler as reported by Peters and van Slyke (1932). Whenever sufficient quantities of serum were not available estimations were made with 0.5 c.c. of serum.

(4) *Diastase*.—The serum remaining after the quantities required for estimations of potassium and inorganic phosphate had been taken was mixed and the diastase content of the mixed serum was estimated by the saccharogenic activity method of Somogyi (1938). Whenever quantities were insufficient estimations were done with serum diluted with 0.5 per cent sodium chloride solution.

(5) *Bilirubin*.—The bilirubin content of mixed serum left after the estimation of diastase was determined by the method of Haslewood and King (1937).

(6) *Ascorbic acid*.—This was determined using Tillman's reduction indicator, 2 : 6 dichlorophenol-indophenol by the method of Birch *et al.* (1933). Precipitation of protein was effected immediately after taking blood.

RESULTS.

A. Blood constituents.

Table I gives the summary of the results of all estimations done with the samples of blood taken from the normal persons and Table II gives that of similar results in diabetic persons.

1. Blood glucose.—(a) Normals :

(i) *Fasting*.—Table I shows that the maximum readings found in the total number examined are from women and the minimum from men. The difference in the average values in the two groups (males and females) is not statistically significant.

The frequency distribution curve for the combined group is given in Graph 1.

Twenty-three out of 31 persons or 74.2 per cent of the results in males, 16 out of 22 or 72.7 per cent of the results in females, i.e. 39 out of 53 or 73.6 per cent of the results in the total number examined, had values between 70 mg. and 100 mg. per 100 c.c. of blood.

Beaumont and Dodds (1939) have given 100 mg. per 100 c.c. as the average fasting blood glucose in a normal person. The values found in the present work are in general agreement with those reported by Glassberg (1931). Gray (1923) obtained for 431 healthy persons values between 80 mg. and 120 mg. per 100 c.c. of blood. The difference between the present values and those reported by other workers may be due to the difference in the analytical procedures.

(ii) *One hour after oral administration of 50 g. of pure dextrose*.—Males had a higher maximum reading and lower minimum under this group (Table I). There was a greater variation in the blood glucose values of males. The difference between the two mean values is significant and the females may be considered to have higher glucose values.

Among males 30 persons (96.8 per cent) had their values between 60 mg. and 160 mg. per 100 c.c. of blood. Seven cases or 22.6 per cent had values between 60 mg. and 80 mg. Only in 16 persons (51.6 per cent) the values were over 100 mg. per 100 c.c. In females all the values were between 80 mg. and 160 mg. per 100 c.c. Thus, compared to the values in males a larger percentage of females had their blood glucose values over 100 mg. per 100 c.c. In the total number examined 13.20 per cent had values between 60 mg. and 80 mg. and 1.9 per cent between 160 mg. and 180 mg. per 100 c.c.

TABLE I.

Number of cases, maximums, minimums, ranges of variation, means, standard deviations and coefficients of variation.
(Normal persons.)

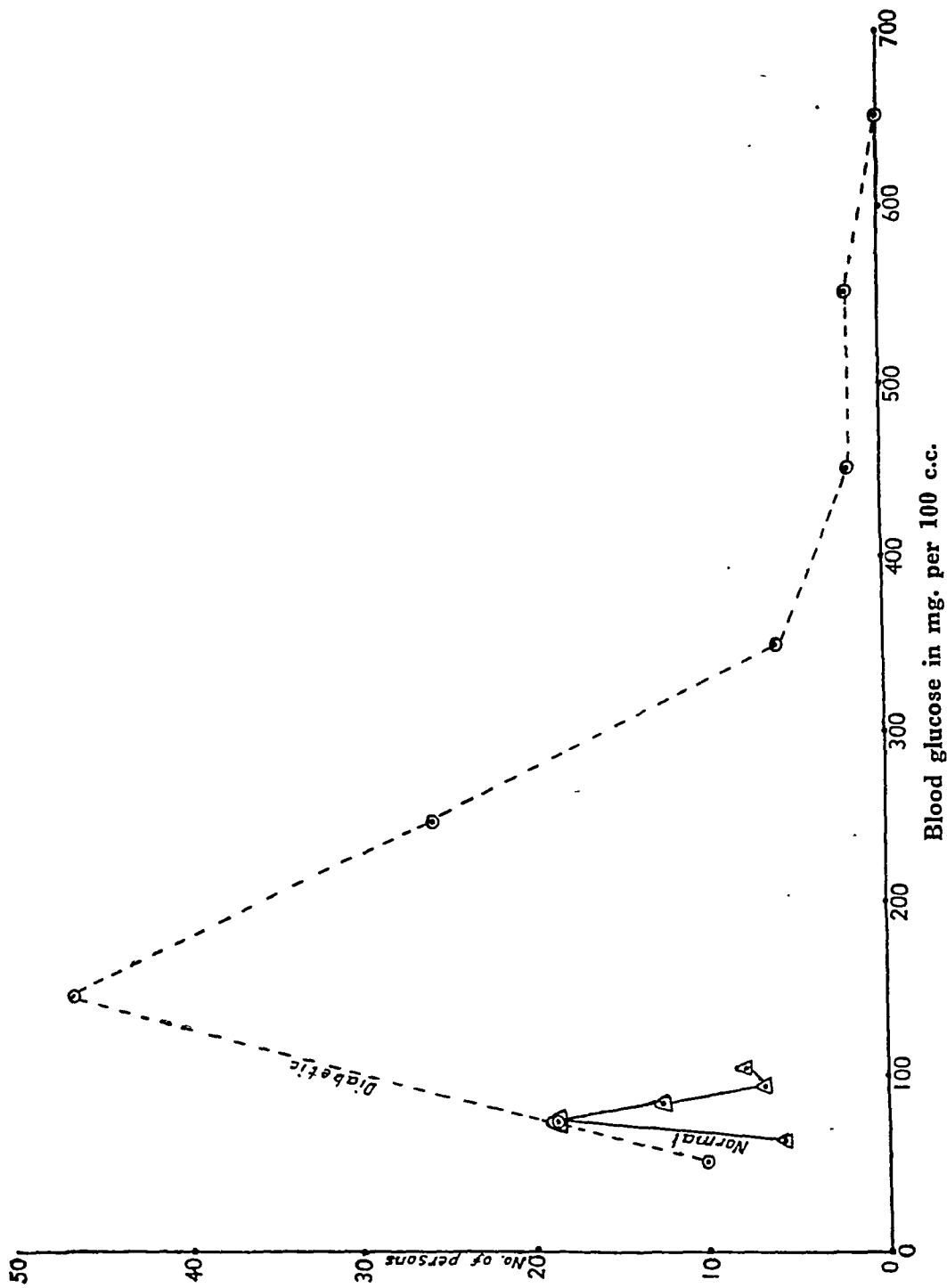
	Group.	BLOOD GLUCOSE.				INORGANIC PHOSPHATE.				POTASSIUM.				Diastase.	Bilirubin.	Ascorbic acid.
		Fasting.	1 hour after.		2 hours after.	Fasting.	1 hour after.	2 hours after.	Fasting.	1 hour after.	2 hours after.					
			Mg. per cent.	Mg. per cent.								Mg. per cent.	Mg. per cent.			
Number of cases.	Males	31	31	31	29	29	29	27	27	27	28	28	30			
	Females	22	22	22	22	21	22	21	22	22	22	22	22			
	Combined	53	53	53	51	48	51	48	48	48	50	49	52			
Maximum	Males	103.10	174.70	135.10	4.495	4.003	4.140	24.30	22.07	23.88	145.0	2.480	2.410			
	Females	108.00	153.80	143.90	5.403	4.761	4.541	24.78	22.78	22.58	148.4	1.401	3.161			
	Combined	108.00	174.70	143.90	5.403	4.761	4.541	24.78	22.78	23.88	148.4	2.480	3.161			
Minimum	Males	61.10	62.60	45.98	2.015	1.820	1.963	13.22	11.16	12.65	45.2	0.292	1.112			
	Females	67.30	90.10	62.81	2.265	2.185	2.100	15.20	15.17	14.54	57.6	0.492	0.948			
	Combined	61.10	62.60	45.98	2.015	1.820	1.963	13.22	11.16	12.65	45.2	0.292	0.948			
Range	Males	42.00	112.10	89.12	2.480	2.183	2.177	11.08	10.91	11.23	99.8	2.188	1.298			
	Females	40.70	63.70	81.09	3.138	2.576	2.441	9.58	7.61	8.04	90.8	0.909	2.213			
	Combined	40.90	112.10	97.92	3.388	2.941	2.578	11.56	11.62	11.23	103.2	2.188	2.213			
Arithmetic mean.	Males	80.89	104.00	82.15	3.076	2.848	2.917	18.89	17.02	17.17	77.5	1.107	1.750			
	Females	86.36	119.09	90.60	3.827	3.282	3.364	19.02	18.21	17.74	89.5	0.795	1.750			
	Combined	83.01	110.11	85.51	3.400	3.099	3.086	18.95	17.46	17.42	83.1	0.959	1.750			
Standard deviation.	Males	±11.77	±26.50	±19.54	±0.630	±0.550	±0.604	±2.97	±2.40	±2.97	±19.7	±0.614	±0.366			
	Females	±15.17	±18.24	±18.26	±0.767	±0.561	±0.661	±3.22	±2.37	±2.30	±21.1	±0.250	±0.688			
	Combined	±12.18	±24.42	±19.18	±0.789	±0.670	±0.655	±2.81	±2.64	±2.65	±21.5	±0.480	±0.518			
Coefficient of variation.	Males	14.55	25.48	23.79	20.47	19.32	20.70	15.72	14.10	17.32	25.42	55.49	20.91			
	Females	17.57	15.31	20.29	20.04	17.09	19.65	16.93	13.01	12.96	23.58	31.45	39.31			
	Combined	14.67	22.18	22.43	23.20	21.62	21.21	14.83	15.12	15.22	25.87	50.05	29.60			

TABLE II.

Number of cases, maximums, minimums, ranges of variation, means, standard deviations and coefficients of variation.
(Diabetic persons.)

Number of cases.	Group.	BLOOD GLUCOSE.			INORGANIC PHOSPHATE.			SERUM POTASSIUM.			Diastase.	Bilirubin.	Ascorbic acid.
		Fasting.	1 hour after.		Fasting.	1 hour after.		Fasting.	1 hour after.				
			Mg. per cent.	Mg. per cent.		Mg. per cent.	Mg. per cent.		Mg. per cent.	Mg. per cent.			
Maximum	Males	84	84	84	61	60	60	62	61	61	66	53	36
	Females	10	10	10	7	7	7	5	5	5	4	7	4
	Combined	94	94	94	68	67	67	67	66	66	70	60	40
Minimum	Males	592.80	707.40	650.40	5.525	5.425	5.285	26.56	25.14	27.75	167.5	2.755	3.236
	Females	289.80	516.10	540.00	4.476	4.114	4.300	19.72	23.23	17.68	111.6	4.318	2.757
	Combined	592.80	707.40	650.40	5.525	5.425	5.285	26.56	25.14	27.75	167.5	4.318	3.236
Range	Males	78.57	144.90	86.12	2.055	2.140	2.055	10.67	10.42	7.63	19.4	0.423	0.488
	Females	96.42	197.90	162.40	2.079	2.910	2.380	14.95	13.91	13.11	39.0	0.556	0.871
	Combined	78.57	144.90	86.12	2.055	2.140	2.055	10.67	10.42	7.63	19.4	0.423	0.488
Arithmetic mean.	Males	514.23	562.50	564.28	3.470	3.285	3.230	15.89	14.72	20.12	118.1	2.332	2.748
	Females	193.38	318.20	377.60	2.397	1.204	1.920	4.77	9.32	4.57	72.6	3.762	1.886
	Combined	514.23	562.50	564.28	3.470	3.285	3.230	15.89	14.72	20.12	148.1	3.895	2.748
Standard deviation.	Males	197.61	310.00	325.00	3.480	3.513	3.424	18.21	17.34	17.22	83.7	1.146	1.511
	Females	191.65	321.83	331.85	3.761	3.761	3.649	16.96	17.26	15.69	70.9	1.475	1.526
	Combined	196.98	310.85	325.37	3.500	3.525	3.435	18.10	17.32	17.12	83.0	1.169	1.513
Coefficient of variation.	Males	102.70	117.10	122.30	0.748	0.734	0.773	3.73	3.58	4.04	34.3	0.525	0.665
	Females	62.61	89.43	112.70	0.836	0.418	0.692	1.73	4.03	1.82	36.6	1.314	0.857
	Combined	98.77	114.20	120.00	0.754	0.710	0.764	3.65	3.69	3.92	34.2	0.659	0.672
	Males	51.92	37.78	37.62	21.49	20.89	22.58	20.48	20.65	23.46	41.0	45.81	44.00
	Females	32.67	27.77	33.94	22.23	11.11	18.96	10.21	23.35	11.60	51.6	89.08	56.16
	Combined	50.13	36.74	36.88	21.54	20.15	22.24	20.15	21.30	22.90	41.2	56.36	44.42

(164)

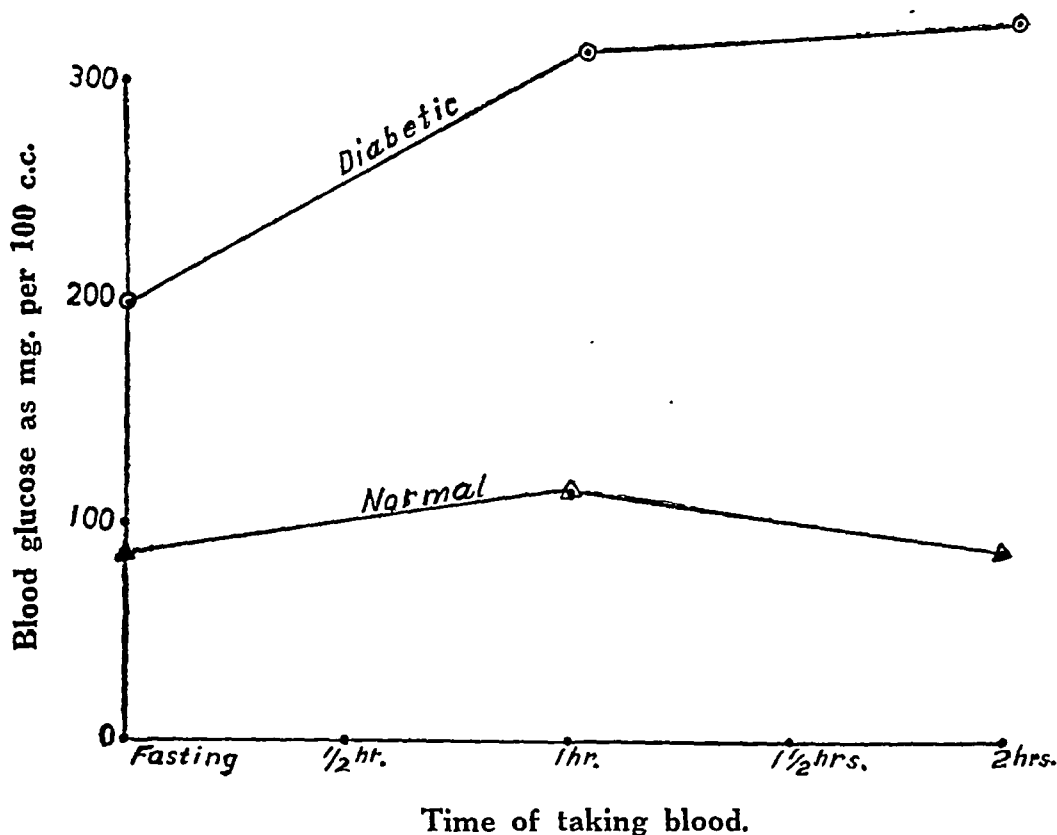


GRAPH 1.—Frequency distribution of glucose values in the fasting blood of (a) diabetics and (b) normals.

(iii) *Two hours after glucose ingestion.*—The maximum reading was in females and the minimum in males (Table I). The variation in blood glucose values found in females is slightly less than those in males. The difference between the mean values of blood glucose in males and females is not significant.

In males three persons (9·7 per cent) had values between 40 mg. and 60 mg. per 100 c.c. and 27 (i.e. 87·1 per cent) between 60 mg. and 120 mg. per 100 c.c. In females there was no person having blood glucose value between 40 mg. and 60 mg. per 100 c.c., while 20 or 90·9 per cent had values between 60 mg. and 120 mg. per 100 c.c. Thus, compared to males a greater percentage of females had values between 60 mg. and 120 mg. per 100 c.c. of blood. In the combined group three (5·7 per cent) had values between 40 mg. and 60 mg. and 47 (88·7 per cent) between 60 mg. and 120 mg. per 100 c.c.

(iv) *Tolerance curves.*—The average blood glucose values of 53 persons examined are given in Graph 2. In 45 cases (84·9 per cent) the middle value, i.e. the value for the glucose content of blood taken one hour after the administration of glucose, was highest. In four (7·6 per cent) they were increasing slightly. In two of these persons the first two values were the same. In one case the middle value was the lowest. In three (5·6 per cent) the values were found to decrease. In one of these three cases the first two values were the same but the third was lower than either of these by 11·7 mg. per 100 c.c.



GRAPH 2.—Mean glucose values in (a) diabetics and (b) normals.

(b) *Diabetics :*

(i) *Fasting* (Table II and Graph 1).—Sixty-four males (76·2 per cent) and 9 females (90·0 per cent) had values between 100 mg. and 300 mg. per 100 c.c. of blood.

(ii) *One hour after administration of 50 g. of pure dextrose.*—Seventy-seven males (92·2 per cent) and 9 females (90·0 per cent) had values between 100 mg. and 500 mg. per 100 c.c.

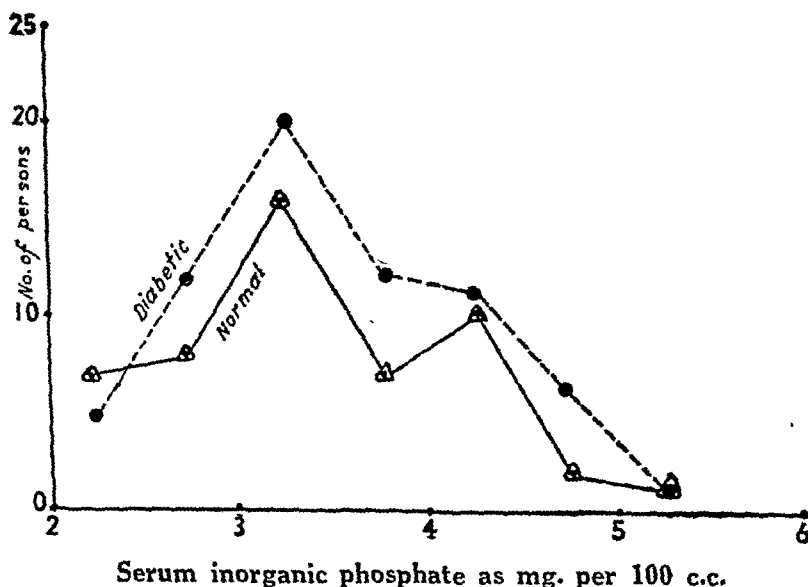
(iii) *Two hours after glucose ingestion.*—Seventy-six males (90·5 per cent) and 9 females (90·0 per cent) recorded values between 100 mg. and 500 mg. per 100 c.c.

(iv) *The tolerance curve.*—Graph 2 shows the curve of average values found in 94 diabetic patients. Thirty-eight or 40·4 per cent of these cases had steadily increasing values. In two of these the last two values were identical; but they were higher than the first. In 56 cases the middle value was the highest. Nine cases (9·6 per cent) had fasting blood glucose values below 100 mg. per 100 c.c. of blood, i.e. their fasting blood glucose values were absolutely normal. Of these, 5 cases had for their maximum slightly below 190 mg. per 100 c.c. and the maximums were reached in one hour. Two of the remaining cases had the maximum values between 190 mg. and 200 mg. and two others over 200 mg. per 100 c.c. These maximums were reached in two hours' time.

2. Serum inorganic phosphate.—(a) Normals :

(i) *Fasting.*—The maximum value was in females (Table I) and minimum in males. The average phosphate value for males is slightly less than that for females in whom the variation was smaller. The difference between the mean values is significant and therefore one can reasonably expect higher serum inorganic phosphate values in females.

The frequency distribution curve for the combined group is given in Graph 3. In males all the values were between 2 mg. and 4·5 mg. per 100 c.c. In 27 (93·2 per cent) the values fell within the range of 2 to 4 mg. per 100 c.c. The distribution of the values in females was slightly different. Only in 19 persons (86·4 per cent) the values were between 2·0 mg. and 4·5 mg. per 100 c.c. In all, 48 persons (94·1 per cent) had phosphate values between 2 mg. and 4·5 mg. per 100 c.c. of serum.



GRAPH 3.—Frequency distribution of inorganic phosphate values in the fasting blood serum from (a) diabetics and (b) normals.

Briggs (1924) found the inorganic phosphate content of human plasmas varying from 2·88 mg. to 3·32 mg. with an average value of 3·04 mg. per 100 c.c. Kay and Byrom (1927) estimated the inorganic phosphate contents of 8 males and 9 females. In males the maximum value was 3·4 mg., minimum value 2·8 mg. and the average value 3·1 mg. per 100 c.c. The corresponding values for females were 3·5 mg., 2·4 mg., and 2·9 mg. per 100 c.c. Peters and van Slyke (1931) found 2 mg. to 5 mg. per 100 c.c. in adults and 4 mg. to 7 mg. per 100 c.c. in infants. Hawk and Bergeim (*loc. cit.*, p. 335) give it as 2 mg. to 5 mg. per 100 c.c. Thus, the values found in the present work show a greater range.

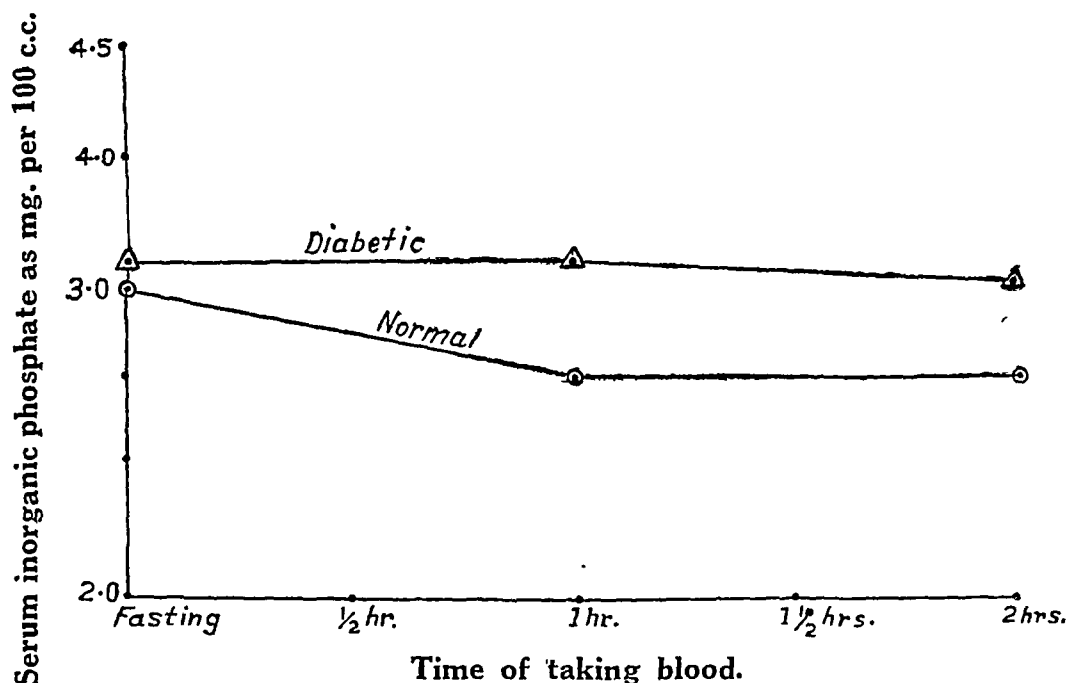
(ii) *One hour after oral administration of 50 g. of glucose.*—The values are higher in females, but the variation in values is greater in males. The difference between the means is significant showing thereby that females generally have a higher inorganic phosphate level than males.

Twenty-eight males (96.6 per cent) had values between 2.0 mg. and 4.5 mg. per 100 c.c. Twenty females (90.9 per cent) and 49 persons or 94.1 per cent of the total number examined were within this range.

(iii) *Two hours after the administration of glucose.*—The maximum was higher, minimum lower, average greater and variation less in the serum inorganic phosphate values of females. The difference between the means in the two groups (males and females) is significant and the phosphate content of serum taken two hours after administration of glucose may be considered to be higher in females than in males.

Out of the 29 males examined for serum inorganic phosphate 28 or 96.6 per cent had values between 2.0 mg. and 4.5 mg. per 100 c.c. In females 21 (95.5 per cent) and in the combined group 49 (96.1 per cent) had values within this range.

(iv) *The tolerance curves.*—The curve of average values of 51 normal persons is given in Graph 4. It will be seen from this curve that one hour after administration of glucose the inorganic phosphate values come to a minimum. However, in three cases the values were found to increase steadily. These ascending values were seen only in males. Descending values were noticed in 28 (54.9 per cent) cases while in 18 (35.3 per cent) the middle value or the value obtained one hour after the administration of glucose was the lowest. In one case the last two values were the same, and in two cases the middle value was the highest.



GRAPH 4.—Mean serum inorganic phosphate values in (a) diabetics and (b) normals.

(b) *Diabetics :*

(i) *Fasting* (Table II and Graph 3).—Fifty-three males (86.9 per cent) and all the females, i.e. 60 patients (88.2 per cent) in all, had values between 2.0 mg. and 4.5 mg. per 100 c.c.

Briggs (1923) found in a diabetic case 3.7 mg. of serum inorganic phosphate. This is within the range of the majority of the values found in the present series.

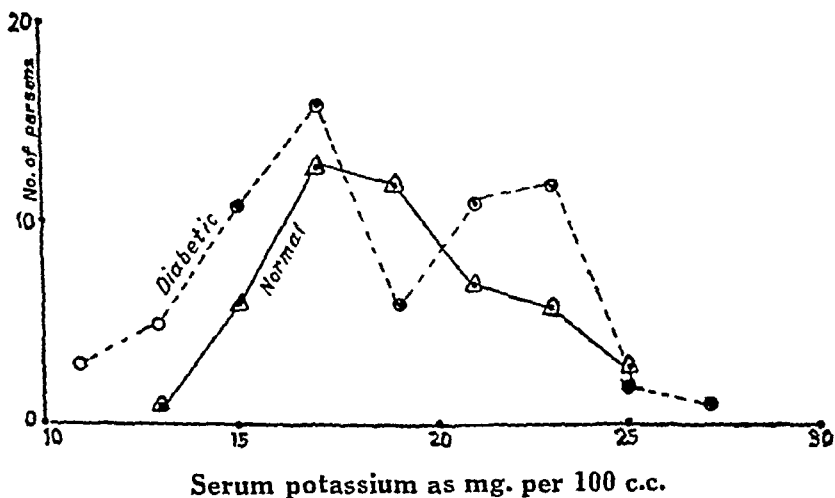
(ii) *One hour after administration of 50 g. of pure dextrose.*—Fifty-five males (91.7 per cent) and all the females had values between 2.0 mg. and 4.5 mg. per 100 c.c.

(iii) *Two hours after glucose ingestion.*—Fifty-four males (90.0 per cent) and all the females had values within the range of 2.0 mg. to 4.5 mg. per 100 c.c.

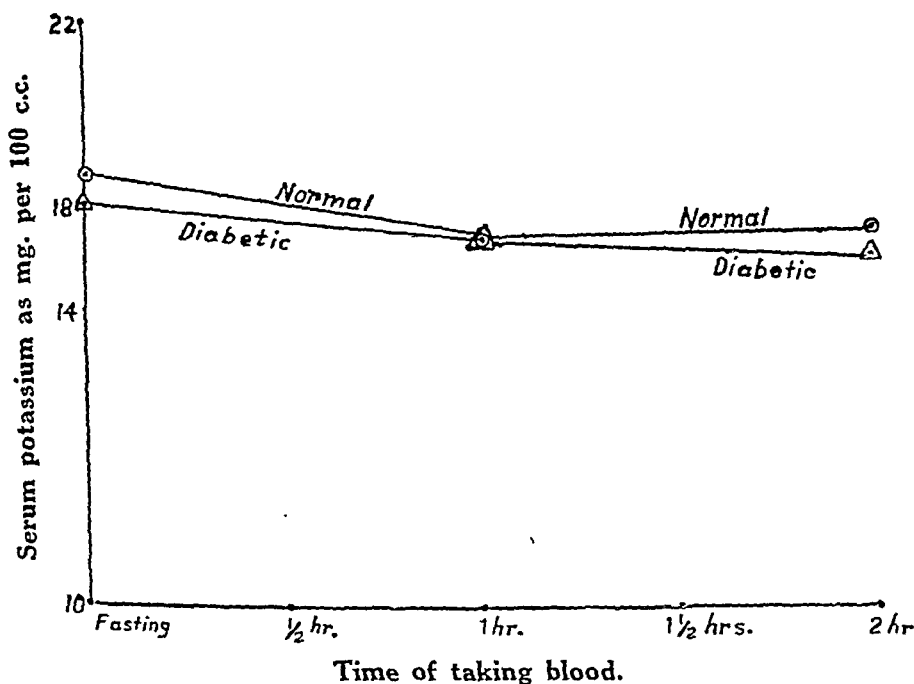
(iv) *The tolerance curves.*—Graph 4 contains the average values of 67 diabetic cases. The general shape of the curve shows a slight rise in the values at the end of one hour and a slight fall at the end of two hours. In 12 patients, i.e. 17.9 per cent of the cases, the values were found to increase, while in 21 or 31.3 per cent, the values were steadily decreasing. In

15 (22.4 per cent) cases the middle values were lowest, while in 19 (28.4 per cent), the middle values were highest.

3. *Serum potassium*.—The frequency distribution curves for combined males and females are plotted in Graph 5 separately for normal and diabetic cases in the fasting condition while Graph 6 contains the tolerance curve.



GRAPH 5.—Frequency distribution of potassium in the fasting serums of (a) diabetics and (b) normals.

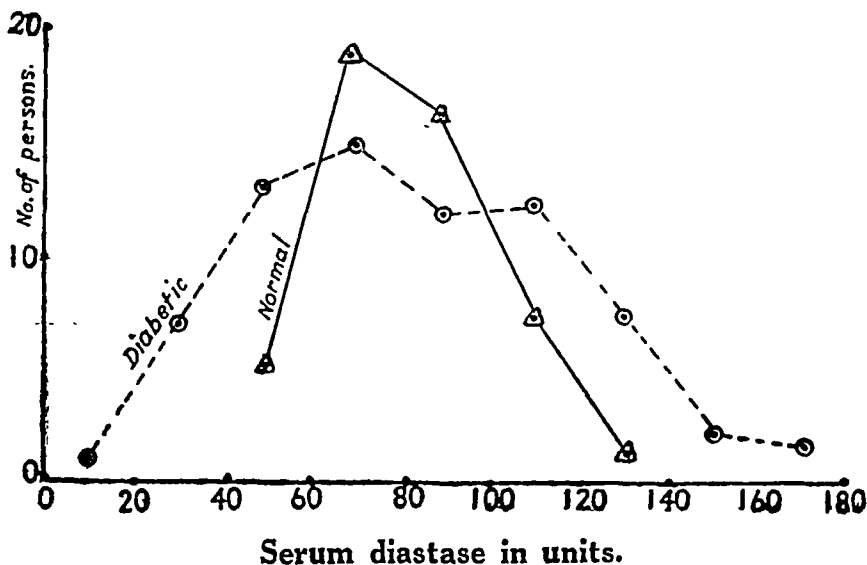


GRAPH 6.—Serum potassium values in (a) diabetics and (b) normals.

(a) *Normal*.—In the fasting condition 96.7 per cent of the cases (92.6 per cent males and 90.5 per cent females) showed values between 14 mg. and 24 mg. per 100 c.c. This range is wider than those already reported in the literature (Taylor, 1930—19.9 mg. to 22.2 mg.; Hawk and Bergeim, *loc. cit.*, p. 474—16 mg. to 22 mg.; Harrison, 1937—18 mg. to 21 mg.). There was no appreciable difference in the values after ingestion of glucose; it will, however, be seen from Graph 6 that there is a fall in values after one hour and a slight rise at the end of two hours.

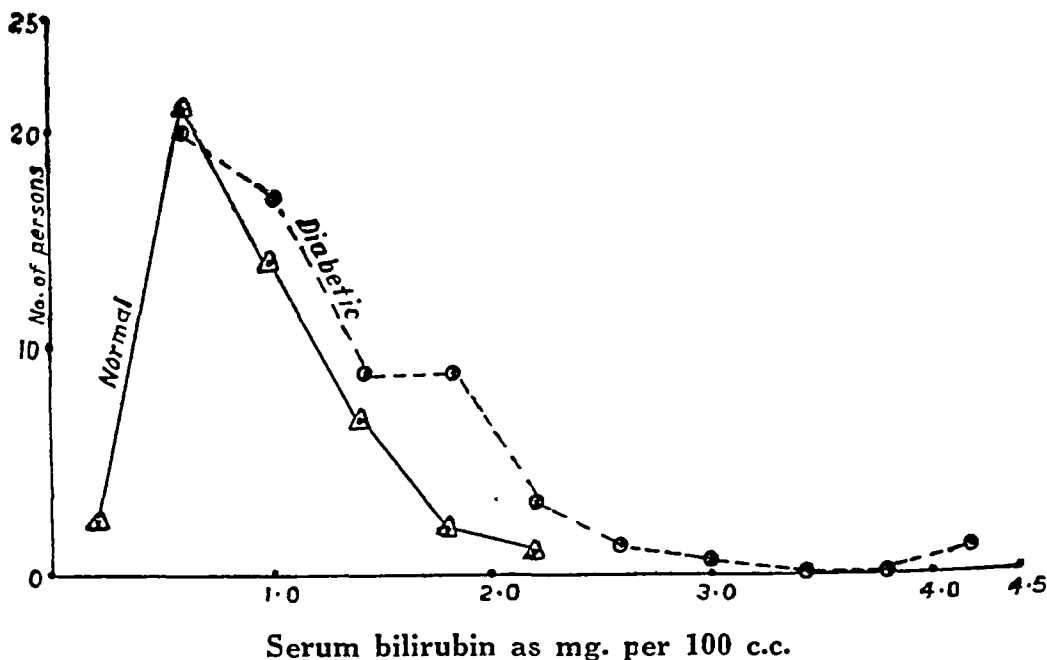
(b) *Diabetics*.—In the fasting condition 91·1 per cent (82·3 per cent males and 100 per cent females) recorded 14 mg. to 24 mg. per 100 c.c. Briggs (1923) reports 23 mg. per 100 c.c. in a diabetic patient. As in the normal cases, glucose ingestion had no effect on serum potassium values, and the lowering in value after one hour is much less pronounced than in the normal cases.

4. *Serum diastase* (Tables I and II, Graph 7).—Eighty-four per cent in normals and 55·7 per cent in diabetics gave values between 60 and 120 units, a range lower than that reported by Somogyi (1942), viz. 80 to 150 units. This difference is probably due to racial differences in the experimental subjects—South Indians and Americans.



GRAPH 7.—Frequency distribution of diastase in the serum of (a) diabetics and (b) normals.

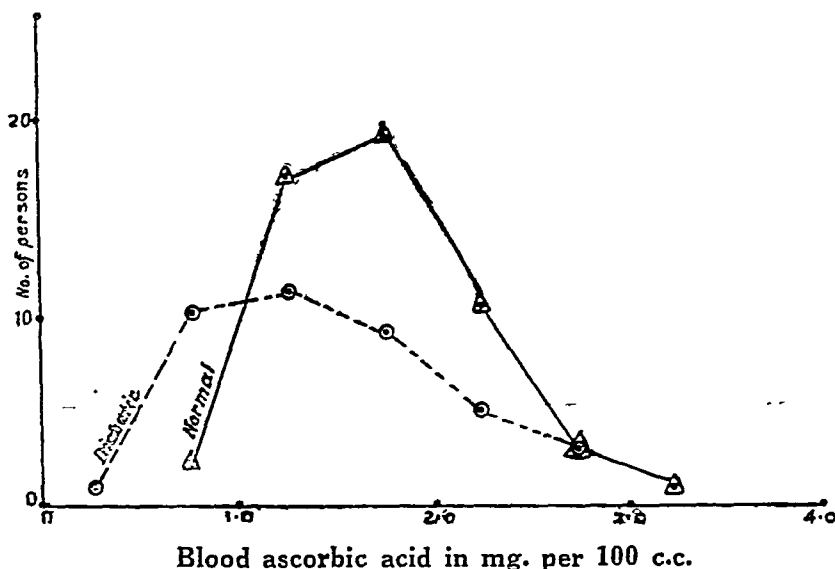
5. *Serum bilirubin* (Tables I and II, Graph 8).—Normal males recorded higher values than corresponding females while diabetic females showed greater variation than diabetic males. The values obtained in this investigation (0·4 mg. to 1·6 mg. per 100 c.c. of serum) are much higher than what has been reported in literature (Hawk and Bergeim, *loc. cit.*, p. 407).



GRAPH 8.—Frequency distribution of bilirubin in the serum of (a) diabetics and (b) normals.

—0.1 mg. to 0.25 mg. per 100 c.c. and Harrison, *loc. cit.*—0.1 mg. to 0.5 mg. per 100 c.c.). Vaughan and Haslewood (1938), however, found the normal level of plasma bilirubin obtained by this method varying from 0.2 mg. to 1.7 mg. per 100 c.c., although most of the values were below 0.8 mg. The results suggest that serum bilirubin of normal South Indians is slightly higher than that of Englishmen.

6. *Blood ascorbic acid* (Tables I and II, Graph 9).—Of normal cases 88.5 per cent and of the diabetic cases 62.5 per cent gave values between 1 mg. and 2.5 mg. per 100 c.c. of blood, a range which indicates that the subjects were not deficient in vitamin C. The figures compare favourably with those reported by other workers (Mirsky *et al.*, 1935—1.11 mg. to 2.88 mg. per 100 c.c.; Bellows, 1936—1.02 mg. per 100 c.c.; Farmer and Abt, 1936—0.69 mg. to 2.36 mg. per 100 c.c.; Hawk and Bergeim, *loc. cit.*, p. 458—0.8 mg. to 2.40 mg. per 100 c.c.; Owen *et al.*, 1941—0.8 mg. per 100 c.c.; Portnoy and Wilkinson, 1938—0.8 mg. to 1.4 mg. per 100 c.c.; Goldsmith and Ellinger, 1939—0.65 mg. to 2.0 mg. per 100 c.c.; Wilder and Wilbur, 1938—0.7 mg. per 100 c.c.; Ralli *et al.*, 1939—1.2 mg. per 100 c.c.).



GRAPH 9.—Frequency distribution of ascorbic acid in the blood taken from (a) diabetics and (b) normals.

B. Correlations.

Table III shows the simple correlation coefficients between the various blood constituents examined (both normal and diabetic) and their significance.

(i) *The relation between fasting blood glucose and fasting serum inorganic phosphate.*—It will be seen from Table III that the correlation coefficients for normals and diabetics are -0.3038 and -0.2536 respectively. These figures and the difference between them are significant, thus establishing a relationship, in diabetes, between fasting serum inorganic phosphate and fasting blood glucose.

The recognized functions of inorganic phosphate are: (i) the maintenance of the acid-base equilibrium of the body, (ii) the formation of bones, (iii) the calcium equilibrium of the body, and (iv) the metabolism of carbohydrates. Henderson (1907) demonstrated that the acid-base equilibrium of the body is contributed to by the conversion of basic phosphates into acid phosphates in the kidney. In acidosis Peters and van Slyke (1931) have shown that the total phosphate excretion is increased and that the transformation of certain types of organic phosphates into inorganic phosphates occurs.

TABLE III.
Coefficients of correlation and their significance.

Between.				COEFFICIENT.	
				Normal.	Diabetic.
1.	Fasting blood glucose and fasting serum inorganic phosphate	+0.3038*	-0.2536*
2.	Relative variation in blood glucose and serum inorganic phosphate	(0 to 1 hour)	...	-0.0902	-0.0586
3.	" " "	(0 to 2 hours)	...	-0.0255	-0.3021†
4.	" " "	(1 to 2 hours)	...	-0.1630	-0.0791
5.	Fasting serum potassium and fasting serum inorganic phosphate	+0.0595	+0.0112
6.	Fasting blood glucose and fasting serum potassium	+0.0138	-0.1203
7.	Relative variation in blood glucose and serum potassium	(0 to 1 hour)	...	+0.0318	-0.0460
8.	" " "	(0 to 2 hours)	...	-0.1280	+0.1592
9.	" " "	(1 to 2 hours)	...	+0.0817	-0.1218
10.	Fasting blood glucose and serum diastase	+0.0259	-0.1600
11.	Fasting blood glucose and serum bilirubin	-0.1727	+0.0615
12.	Serum bilirubin and serum diastase	+0.1123	+0.0954
13.	Fasting blood glucose and blood ascorbic acid	-0.1559	+0.0873

* Significant at 5 per cent level.

† Significant at 1 per cent level.

It is generally recognized (Schmidt and Greenberg, 1935) that from the chemical standpoint the skeleton is not a fixed system, but one in which there is a frequent interchange of inorganic constituents, especially calcium, phosphorus and magnesium. In fact, the skeleton constitutes a great reservoir of these ions which can be readily called on in times of need.

After the administration of insulin (Blatherwick *et al.*, 1924; Bollinger and Hartman, 1925; Briggs *et al.*, 1924; Harrop and Benedict, 1924; Kay and Robinson, 1924; Sokhey and Allen, 1924), glucose (Bollinger and Hartman, *loc. cit.*) and adrenaline (Sacks, 1927; Vollmer, 1923) the inorganic phosphate of the blood and urine falls. Hartman and Bollinger (1925) found that in severe experimental and clinical diabetes the administration of glucose does not affect phosphate metabolism. Peters and van Slyke (1931) suggested that, as the phosphate reserves were adequate for carbohydrate metabolism, the variation in blood phosphate did not influence glucose combustion. They stated that, in diabetes, the changes in plasma phosphate which normally follow the ingestion of carbohydrate were delayed, diminished or were absent and that the changes depend on the degree of carbohydrate tolerance. Hartman and Bollinger (*loc. cit.*) suggested that post-alimentary phosphaturia curves could be used as an index of the severity of diabetes. In severe cases of diabetic acidosis Peters *et al.* (1925) found slight reduction in serum inorganic phosphate content.

In the present work blood glucose was found to have a significant correlation with serum inorganic phosphate. In normal persons the relationship is direct, i.e. in cases where fasting blood glucose is high, the fasting serum inorganic phosphate is also high. This is because both these constituents, which are required for carbohydrate metabolism, are easily mobilized. In diabetes the metabolism of glucose is deranged and to ensure a normal utilization of glucose a higher glucose pressure is maintained.

In normal cases where there is no acidosis or polyuria the mechanism for the retention of bases may be sufficient. But, when there is polyuria and acidosis or polyuria alone, the excretion of phosphate is increased so that an increased production of phosphate is essential. Rabinowitch (1924) found that diabetics with nephritis could not produce as much ammonia as normals. Assuming that the liberation of phosphate is normal and the urinary excretion increased, it is only reasonable to expect a lowered phosphate content in the serum.

Further, serum inorganic phosphate is in equilibrium with the phosphate reserve from which the former is mobilized irrespective of the form in which this reserve exists. The fact that an inverse relationship exists between serum inorganic phosphate and blood glucose in diabetic cases shows that the mechanism which produces increased phosphorus content in normal cases is deranged in diabetic cases. It cannot be said that excretion alone can be held responsible for this, for if there is greater excretion there can also be greater mobilization. Further, there is some relationship between blood glucose values and serum inorganic phosphate values. We have, therefore, to infer that in diabetic cases either there is some derangement in the mobilization mechanism, or with the raised level of blood glucose an increased quantity of inorganic phosphate is used up to make the utilization of glucose the same.

(ii) *Relation between the relative variations in blood glucose and serum inorganic phosphates noted in the first two hours after the administration of glucose.*—The coefficients of correlation for normals and diabetics are -0.0255 and -0.3021 respectively. The difference is not significant, and so, as far as the relations between relative changes in the values of blood glucose and serum inorganic phosphate are concerned, the diabetics cannot be considered as different from normals.

Peters *et al.* (*loc. cit.*) occasionally noticed a slight reduction of the inorganic phosphate of serum in cases of severe diabetic acidosis. In the present work a negative correlation between glucose and phosphate values is noticed in the normal as well as in diabetic cases. The result is not significant in the normal while it is highly significant in diabetic cases; and as far as this relationship is concerned the normal persons are not in any way different from diabetic patients. In other words, in all types of cases we can expect to find a lowering in the phosphate values in the first two hours.

It is, however, to be noted that if only average values are taken, as in the case of tolerance curves, the lowering in the phosphate values after the administration of glucose is greater in normal than in diabetic cases. In diabetics, the curve of the average values run almost parallel to the abscissa showing thereby that an oral administration of glucose does not materially affect the serum inorganic phosphate values in diabetes. These results are in agreement with the findings of the previous workers.

No other significant relationship was observed between the various constituents estimated.

SUMMARY AND CONCLUSIONS.

1. The glucose and ascorbic acid contents of blood and inorganic phosphate, potassium, bilirubin and diastase contents of serum were determined in 53 normal persons (31 males and 22 females) and 94 diabetic patients (84 males and 10 females).

2. The relationship between fasting serum inorganic phosphate and fasting blood glucose is definitely altered in diabetes.

3. There is significant inverse relationship between the relative variations in the glucose content of blood and inorganic phosphate content of serum during the first two hours after the administration of glucose in the case of diabetic subjects. However, diabetics cannot be considered different from normals in this respect.

4. There is no evidence to show that the relationships between the other blood constituents examined are altered in diabetes.

The authors record here their thanks to other members on the staff of the Biochemistry Department of the Medical College, Madras, for their co-operation and help, to the Superintendents of the various Government Hospitals in Madras for their permission to take

the necessary notes from the case sheets of patients on whom the above research work was conducted, to Dr. N. Sundararama Sastri for his suggestions regarding the statistical treatment of the values and to the volunteers, in the control experiments, for their co-operation.

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STATISTICAL STUDIES IN GLUCOSE TOLERANCE.

Part I.

BLOOD GLUCOSE OF NORMAL MALE SUBJECTS.

BY

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A CLINICAL determination of glucose tolerance is recognized to be of fundamental diagnostic and therapeutic value in pathological conditions, particularly diabetes. The interpretation of the results is based on a set of so-called normal values. However, the normal range itself is rather wide even when the study is confined to a specific cross-section of a country or community. This fluctuation cannot be attributed to the lack of adequate physiological regulatory mechanisms, for it is well known that the human body is capable of exercising highly sensitive control, e.g. maintenance of pH of blood, temperature, etc. It would appear, therefore, that the normal variation in blood glucose is not entirely fortuitous and some work was, therefore, undertaken to study the relation, if any, between blood glucose on the one hand, and surface area and age of normal and pathological subjects on the other. The results obtained with normal male subjects are reported in this paper. Similar figures for females, and for pathological cases, will form the subject of subsequent communications.

MATERIAL AND METHODS.

Twenty healthy subjects were chosen from the students and staff of the Medical College, Madras, for this study. Age was calculated correct to the second decimal place from records of date of birth and date of experiment. Body-surface area was calculated from the height and weight of the individual according to the surface-area nomogram method of Boothby and Sandiford (quoted by Hawk and Bergeim, 1938). Blood glucose was determined prior to, and at fixed intervals of $\frac{1}{2}$ hour, 1 hour, $1\frac{1}{2}$ hours and 2 hours after administration of 50 g. of pure dextrose. Determinations were done in duplicate according to the colorimetric method of Herbert and Bourne (1931). Urine samples were tested to ensure absence of abnormal amounts of glucose.

RESULTS.

The detailed results are given in Table IV. Table I shows the general nature of the results obtained.

It will be seen that wide variations in glucose values have been recorded. On purely statistical grounds, values within the arithmetic mean \pm twice the standard deviation are likely to occur in 95 per cent of the cases, and by this criterion alone values much lower than 70 mg. of glucose per 100 c.c. of blood should normally be encountered. That this is rarely the case is proof of the intervention of physiological regulatory mechanisms.

The relationship between the fasting blood glucose values on the one hand and the body-surface area and the age on the other in males between the ages of 18 and 32 is given by the regression equation :—

$$\text{Fasting blood glucose in mg. per cent} = -14.12034 + 70.49996 \times \text{body-surface area in square metres} - 0.84104 \times \text{age in years.}$$

TABLE I.

	Height in inches.	Weight in pounds.	Surface area in square metres.	Age in years.	BLOOD GLUCOSE VALUES IN MG./100 C.C.				
					Fasting.	$\frac{1}{2}$ hour after.	1 hour after.	$1\frac{1}{2}$ hours after.	2 hours after.
Number examined ...	20	20	20	20	20	20	20	20	20
Maximum ...	73.3	169.0	1.99	31.83	103.10	156.20	150.95	146.00	119.35
Minimum ...	62.0	102.0	1.44	18.35	61.10	81.30	71.20	69.80	45.98
Range ...	11.3	67.0	0.55	13.48	42.00	74.90	79.75	76.20	73.37
Arithmetic mean ...	66.5	126.4	1.64	23.38	82.01	107.51	100.11	89.87	81.09
Standard deviation	± 2.94	± 19.38	± 0.16	± 4.06	± 13.11	± 18.49	± 24.65	± 19.14	± 17.37
Coefficient of variation	4.43	15.33	9.50	17.36	15.99	17.20	24.62	21.25	21.42

The coefficient + 70.49996 is very highly significant (significant even at 0.3 per cent level) showing thereby that the body-surface area profoundly influences the blood glucose level and that the relationship is direct, i.e. the larger the surface area the higher will be the fasting blood glucose values. This direct relationship exists between these probably because of the greater radiations from the body. The factor connecting fasting blood glucose values and age (between 18 and 32 years) is negative and is not significant at 5 per cent level. The two factors are, therefore, correlated inversely, i.e. within this age limit, as the age advances blood glucose values decrease. However, the magnitude of the coefficient being not significant statistically evidence is not sufficient to say that this relationship exists. For convenience the fasting blood glucose values of males from 16 years to 32 years having body-surface area from 1.25 to 2.12 square metres are calculated and given in Table II:—

TABLE II.

Blood glucose values in normal males between the ages of 16 and 32 years.

(Values as mg. per 100 c.c. of blood.)

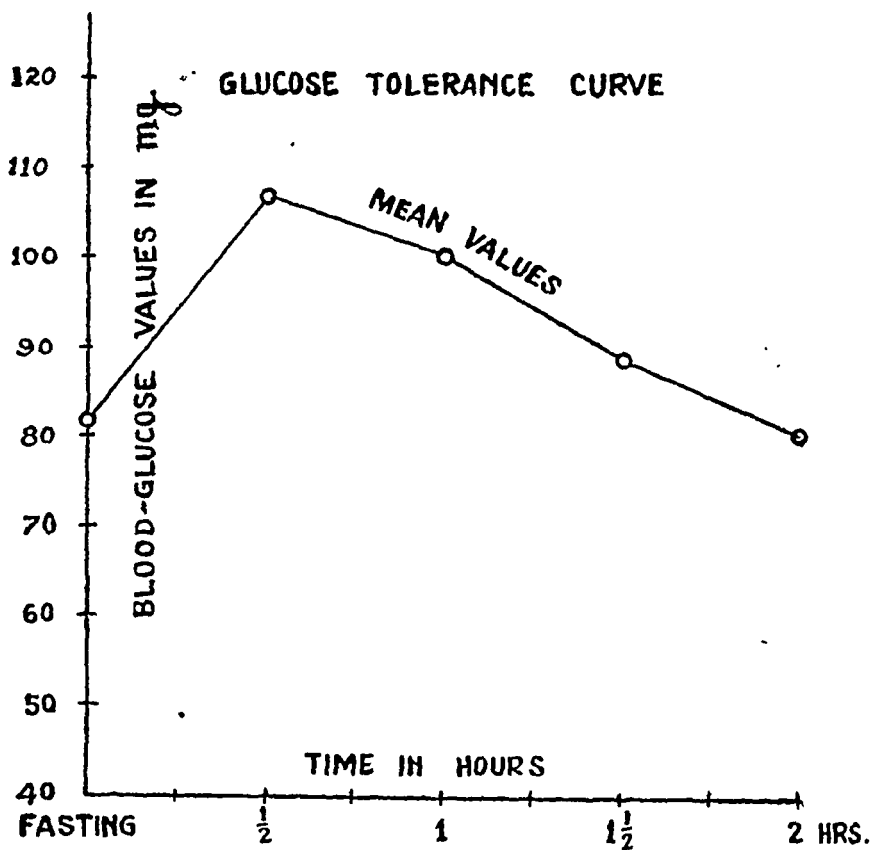
Body-surface area in square metres.	Age in years.								
	16	18	20	22	24	26	28	30	32
1.25	60.548	58.866	57.184	55.502	53.820	52.138	50.455	48.773	47.091
1.28	62.663	60.981	59.299	57.617	55.935	54.253	52.570	50.888	49.206
1.31	64.778	63.096	61.414	59.732	58.050	56.368	54.685	53.003	51.321
1.34	66.893	65.211	63.529	61.847	60.165	58.483	56.800	55.118	53.436

TABLE II—*concl'd.*

Body-surface area in square metres.	Age in years.								
	16	18	20	22	24	26	28	30	32
1·37	69·008	67·326	65·644	63·962	62·280	60·598	58·915	57·233	55·551
1·40	71·123	69·441	67·759	66·077	64·395	62·713	61·030	59·348	57·666
1·43	73·238	71·556	69·874	68·192	66·510	64·828	63·145	61·463	59·781
1·46	75·353	73·671	71·989	70·307	68·625	66·943	65·260	63·578	61·896
1·49	77·468	75·786	74·104	72·422	70·740	69·058	67·375	65·693	64·011
1·52	79·583	77·901	76·219	74·537	72·855	71·173	69·490	67·808	66·126
1·55	81·698	80·016	78·334	76·652	74·970	73·288	71·605	69·923	68·241
1·58	83·813	82·131	80·449	78·767	77·085	75·403	73·720	72·038	70·356
1·61	85·928	84·246	82·564	80·882	79·200	77·518	75·835	74·153	72·471
1·64	88·043	86·361	84·679	82·997	81·315	79·633	77·950	76·268	74·586
1·67	90·158	88·476	86·794	85·112	83·430	81·748	80·065	78·383	76·701
1·70	92·273	90·591	88·909	87·227	85·545	83·863	82·180	80·498	78·816
1·73	94·388	92·706	91·024	89·342	87·660	85·978	84·295	82·613	80·931
1·76	96·503	94·821	93·139	91·457	89·775	88·093	86·410	84·728	83·046
1·79	98·618	96·936	95·254	93·572	91·890	90·208	88·525	86·843	85·161
1·82	100·733	99·051	97·369	95·687	94·005	92·323	90·640	88·958	87·276
1·85	102·848	101·166	99·484	97·802	96·120	94·438	92·755	91·073	89·391
1·88	104·963	103·281	101·599	99·917	98·235	96·553	94·870	93·188	91·506
1·91	107·078	105·396	103·714	102·032	100·350	98·668	96·985	95·303	93·621
1·94	109·193	107·511	105·829	104·147	102·465	100·783	99·100	97·418	95·736
1·97	111·308	109·626	107·944	106·262	104·580	102·898	101·215	99·533	97·851
2·00	113·423	111·741	110·059	108·377	106·695	105·013	103·330	101·648	99·966
2·03	115·538	113·856	112·174	110·492	108·810	107·128	105·445	103·763	102·081
2·06	117·653	115·971	114·289	112·607	110·925	109·243	107·560	105·878	104·196
2·09	119·768	118·086	116·404	114·722	113·040	111·358	109·675	107·993	106·311
2·12	121·883	120·201	118·519	116·837	115·155	113·473	111·790	110·108	108·426

The Graph represents the average glucose values. The maximum is reached in $\frac{1}{2}$ hour after the administration of glucose and the minimum in 2 hours.

GRAPH.



The coefficients of correlation between fasting blood glucose and body-weight, body-surface area and the blood glucose values at different times after administration of pure dextrose and the levels of significance are given in Table III :—

TABLE III.

Correlation coefficients and levels of their significance.

Between	Correlation coefficient.	Levels of significance (as per cent).
1. Fasting blood glucose and body-weight ...	0.5971	0.7
2. „ and body-surface area ...	0.6478	0.3
3. „ and blood glucose after $\frac{1}{2}$ hour	0.5133	2.1
4. „ „ 1 hour	0.6180	0.6
5. „ „ $1\frac{1}{2}$ hours	0.5250	1.8
6. „ „ 2 hours	0.7496	Below 0.1

It will be seen from this that the correlation between fasting blood glucose and body-weight is significant at 0.7 per cent level and that between the former and body-surface

area is significant even at 0.3 per cent. This shows that while both body-weight and body-surface area can alter the values of the fasting blood glucose, such variations due to changes in surface area are greater than those due to changes in body-weight alone.

Table III also shows that the blood glucose values noted after the oral administration of glucose vary directly with the fasting blood glucose values. All the coefficients of correlation are significant. The values after 1 hour and 2 hours are significant even at 1 per cent and 0.1 per cent respectively showing thereby that the influence of the level of the fasting blood glucose on the variations in the blood glucose values at 1 hour and 2 hours is exhibited to a greater degree.

TABLE IV.

Values in males between the ages of 18 and 32 years.

Serial number.	Height in inches.	Weight in pounds.	Body-surface area in square metres.	Age in years.	BLOOD GLUCOSE VALUES IN MG./100 C.C.				
					Fasting.	$\frac{1}{2}$ hour after.	1 hour after.	$1\frac{1}{2}$ hours after.	2 hours after.
1	67.0	124.0	1.64	31.83	85.80	95.20	91.30	90.50	90.90
2	63.5	122.0	1.57	23.03	86.90	156.20	106.90	123.50	89.50
3	64.5	159.5	1.77	21.73	87.10	101.00	111.70	88.70	94.30
4	67.0	112.0	1.58	22.88	77.65	121.90	148.10	114.30	93.90
5	63.0	118.0	1.54	23.39	64.30	85.80	85.50	83.85	58.00
6	68.0	136.0	1.73	26.70	71.30	93.65	88.70	84.70	62.80
7	68.5	150.0	1.81	30.13	86.40	100.00	86.40	86.00	88.10
8	64.3	120.0	1.56	21.97	82.08	87.08	100.50	85.65	75.39
9	66.0	127.0	1.64	21.72	71.55	99.60	61.50	66.20	59.30
10	62.5	102.0	1.44	20.53	69.00	101.50	89.10	87.70	66.20
11	64.3	102.0	1.46	19.43	68.70	125.40	107.50	83.30	90.90
12	67.5	118.0	1.61	25.42	61.10	86.90	62.60	66.80	45.98
13	68.0	130.8	1.70	21.47	103.10	113.60	99.80	87.70	93.00
14	71.5	159.0	1.91	30.77	103.10	122.70	129.00	146.00	119.35
15	63.0	110.0	1.49	20.47	77.95	101.50	97.10	82.15	94.90
16	62.0	105.0	1.45	21.32	74.20	81.30	78.40	69.80	69.10
17	68.0	121.0	1.65	19.53	75.85	107.20	71.20	78.70	71.40
18	68.8	129.0	1.70	18.35	102.85	120.50	117.00	84.00	90.50
19	68.8	114.0	1.61	21.87	91.70	124.60	119.00	84.20	76.75
20	73.3	169.0	1.99	25.08	99.60	124.60	150.95	103.60	91.50

DISCUSSION.

The results given above indicate that the variations in the normal values are not without any purpose, but represent a definite and scientific arrangement for the maintenance of the

energy supply necessary for the human machine and that perfect order exists in these variations. It is, therefore, imperative to eliminate the influence of this factor (due to body-weight or surface area) in studies of blood glucose values if a greater insight into the nature of the variations in the blood glucose values in the various pathological conditions is desired. The variations found in the blood glucose values in cases of derangement of carbohydrate metabolism (diabetes) are so great that even without the actual calculation of normal blood glucose values one can diagnose or treat a diabetic case. However, to recognize the borderline cases and for further study of the causes of derangement of the fine mechanisms existing in the human system we hope this finding will be of some definite help. Administering glucose according to the body-weight has been recognized before, but we feel that the body-surface area and not the body-weight should be taken into account since in metabolism experiments it is the former that is found to influence the findings. Further, the significance is greater in samples taken 1 hour and 2 hours after oral administration of glucose. The greater the interval between the oral administration of glucose and the time of taking blood the greater will be the relationship between fasting blood glucose level and changes in the blood glucose values. This suggests that after a particular time the changes in blood glucose values will be governed entirely by the height of the fasting blood glucose level.

SUMMARY.

1. Statistical analysis of glucose values of fasting blood from 20 normal male subjects between the ages of 18 and 32 shows (i) that the glucose level of fasting blood is affected by variations in body-surface area, but not by those in age and (ii) that there is a high degree of correlation between the glucose levels of blood taken before and after administration of glucose.

2. A multiple regression formula of blood glucose on body-surface area and age is given with a table of estimated glucose values.

The authors wish to thank Dr. Y. C. Chellappa, Mr. P. S. B. Pillai and Mr. Y. V. N. Ayya for their kind help and useful suggestions and the volunteers (subjects of this paper) for their co-operation.

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PHARMACOLOGICAL ACTION OF AN ACTIVE CONSTITUENT
ISOLATED FROM *DÆMIA EXTENSA* LINN.
(*SYN. PERGULARIA EXTENSA*).

Part I.

BY

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INTRODUCTION.

Dæmia extensa is a perennial twining herb of the N. O. *Asclepiadaceæ*. It occurs throughout the hotter parts of India, and is known as 'Châgulgânti' in Bengali and 'Uttaravaruni' in Sanskrit. It is a drug of good repute in the Ayurveda, and amongst its multifarious therapeutic uses, mention may be made of its use in uterine complaints and to facilitate parturition. The plant is said to contain a very bitter glucoside. As this drug is extensively used in Hindu medicine, we have studied its pharmacological action and present the results in this paper.

CHEMISTRY.

For our investigation, the entire plant, including root, leaves, stem, pods, etc., was used. One kilogram of the air-dried plant was coarsely powdered and thoroughly extracted with rectified spirit. After removing the oily matter with petroleum ether, the dried mass was treated with hot amyl alcohol. The solvent was then removed, and the residue successively treated with ether, chloroform and ethyl acetate. The final product was found to contain a principle of glucosidic nature, having marked physiological activity; this was dried and further purified till the glucoside was obtained in a fairly pure condition. A fresh 1 per cent aqueous solution of this principle, which gave fairly uniform results in preliminary experiments, was used in all the experiments mentioned below.

Besides this principle, the plant also contains other bitter substances, potassium salts, oils, resin, sterol and some reducing sugar.

PHARMACOLOGY.

The effect of the drug on different organs was studied on frogs, guinea-pigs and cats. Toxicity experiments were done on frogs, mice and rats.

Toxicity.—35 mg./kg. was found to be the lethal dose in frogs weighing between 250 g. and 300 g.; and that in white mice of average weight 20 g. to 25 g. was 12.5 mg./kg.

Alimentary system.

The intestines.—0.15 mg./kg. to 0.75 mg./kg. injected intravenously in the cat increased the tone and movements of the intestines *in situ*. This effect disappeared to some extent, though not completely, after administration of atropin. In isolated sections of intestine of

cat, kitten and guinea-pig also, the tone and movement increased even with such dilutions of the drug as 1 in 500,000 (see Graph 1, fig. 6).

Cardiovascular system.

The heart: (1) *Mammalian heart in situ.*—0.35 mg./kg. to 0.5 mg./kg. given intravenously in the cat increased the force of contractions of both auricles and ventricles; slight slowing of the ventricular rhythm with prolonged diastole was noticed. After atropin, the force of contractions increased as before, but there was no slowing of the rhythm. With toxic doses the heart stops in diastole.

(2) *Excised frog heart:* (a) *Force and rhythm.*—Slight stimulation of beats were noticed even in a dilution of 1 in 300,000,000, though the rhythm remained practically unaltered; slowing of the rhythm could, however, be noticed in dilution of 1 in 1,500,000. In lower dilutions, the force of beats gradually increased while the rhythm gradually slowed, till in a dilution of 1 in 300 the beats became irregular and intermittent, but more and more forcible, and the heart ultimately stopped in diastole (Graph 1, fig. 5).

(b) *Output.*—In a dilution of 1 in 100,000 the output increased appreciably; in lower dilutions the output increased further.

Blood vessels.—Frog's blood vessels perfused by Bain's method showed that 0.5 mg. decreased the rate of outflow slightly. In higher doses it produced more pronounced results. After atropin, the rate of outflow decreased further; but after ergotoxin the rate remained practically as before.

Blood pressure.—0.1 mg./kg. to 1.45 mg./kg. given intravenously in the cat almost invariably caused a rise of blood pressure. In some instances an initial fall, quickly reversed, was noticed. In certain other cases, a secondary rise higher than the first was observed. The rise of pressure did not appear to be proportional to the dose used. With a moderate dose, 0.3 mg./kg. to 0.5 mg./kg., a rise of about 20 to 30 per cent of the original, maintained for some time, was generally noticed. After ergotoxin, the percentage of rise was higher; after atropin, it was much higher; and after section of both the vagi in the neck, it was higher still, though less than after atropin. In the spinal cat, on the other hand, the percentage of rise after atropin was only slightly higher, while in the decerebrated cat, after ergotoxin, it was actually lower (Graph 1, figs. 1, 2, 3 and 4).

Spleen volume.—The volume of the spleen decreased while the blood pressure was increasing. This response did not alter even after atropin or ergotoxin (Graph 1, figs. 1, 2, 3 and 4).

Limb volume.—The peripheral limb volume increased with the arterial pressure; but its fall did not correspond with the fall of the pressure. After atropin, no change in the volume was noticed.

Genito-urinary system.

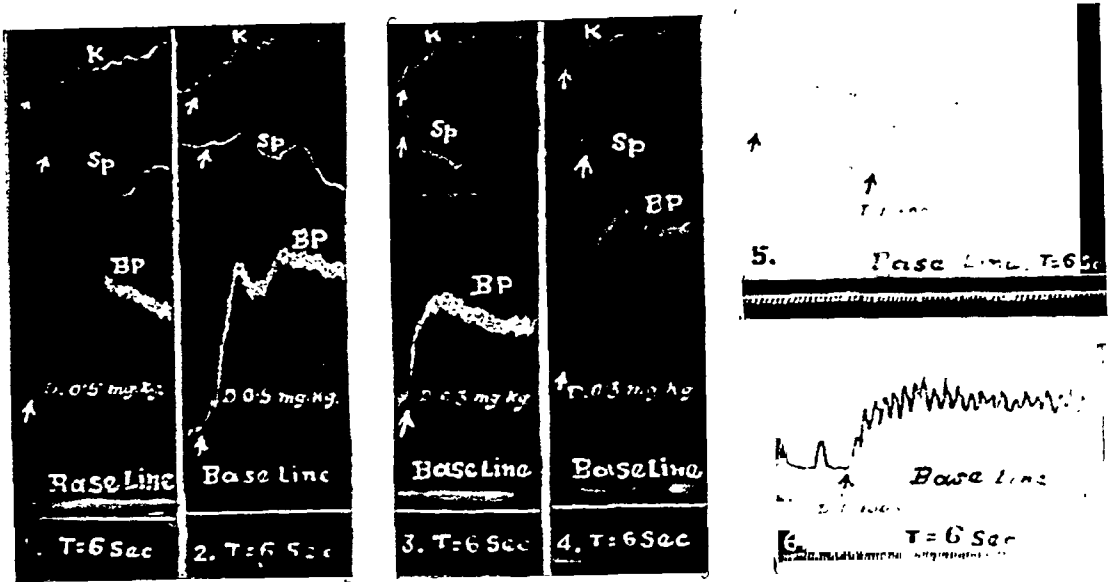
(1) *The kidney.*—The volume of the kidney increased with the rise of the blood pressure; the increase persisted even when the pressure decreased. After atropin and ergotoxin, the volume practically followed the course of the blood pressure (Graph 1, figs. 1, 2, 3 and 4).

(2) *The urinary bladder.*—Intravenously 0.10 mg./kg. increased the tone and movements of the urinary bladder in the cat. After atropin, the movements became slightly sluggish. Higher doses tended to produce relaxation of movements.

(3) *The uterus:* (a) *Uterus in situ.*—Increased tone and contractions were observed in both the pregnant and the non-pregnant uterus of the cat, *in situ*, in doses of 0.05 mg./kg. to 1.0 mg./kg. intravenously (Graph 2, figs. a and c).

(b) *Excised uterus.*—The excised non-pregnant uterus of the guinea-pig responded by increased tone and contractions in dilution from 1 in 50,000 to 1 in 200,000. After treatment in a bath of atropin sulphate, 1 in 100,000 for 20 to 30 minutes, the contractions were smaller (Graph 2, figs. e, f and g).

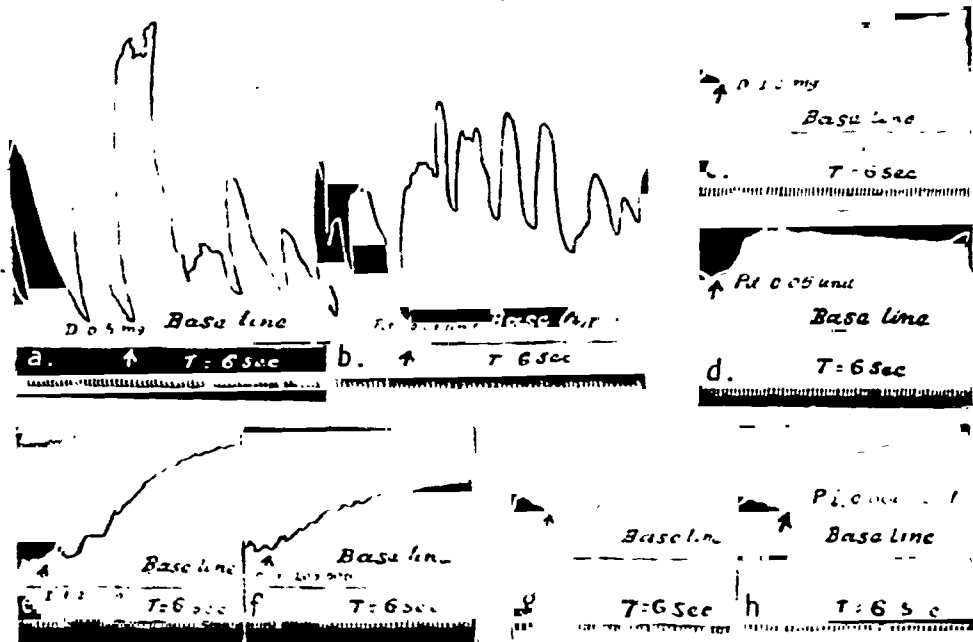
GRAPH 1.



Effect of dæmia on

- 1 and 2.—Cat's carotid blood pressure, kidney and spleen volumes : before and after atropin.
- 3 and 4.—The same : before and after ergotoxin.
- 5.—Isolated frog heart.
- 6.—Isolated pieces of cat's intestines.

GRAPH 2.



- a and c.—Effect of dæmia on pregnant and virgin uterus of cat respectively (in situ).
- b and d.—Effect of pituitrin on the same. e and f.—Effect of dæmia on isolated virgin uterus of guinea-pig: before and after atropin. g and h.—Effect of dæmia and pituitrin on isolated virgin uterus of guinea-pig respectively.

Action of dæmia on the uterus as compared with that of pituitrin.

The pregnant and the non-pregnant uterus of the cat, *in situ*, and the excised virgin uterus of the guinea-pig were all observed to have their tone and contractions augmented with dæmia and pituitrin. As regards the virgin uterus of the cat, *in situ*, the maximum contraction produced by 0.05 units of pituitrin was practically the same as by 1.0 mg. of dæmia, both being given intravenously. The non-pregnant uterus of the multiparous cat contracted less with 0.50 mg. of dæmia, but more with 1.0 mg., than with 0.025 units of pituitrin, all given intravenously. In the pregnant cat, 0.50 mg. of dæmia intravenously produced a much higher contraction than 0.01 unit of pituitrin administered similarly. The relaxation which followed was much more complete after dæmia than after pituitrin, so that the tone after pituitrin was higher than after dæmia. The frequency of contractions was also higher after pituitrin than after dæmia. The excised virgin uterus of the guinea-pig responded practically to the same extent to 0.0001 unit of pituitrin as to a concentration of dæmia of 1 in 200,000 (Graph 2, figs. a, b, c, d, g and h).

DISCUSSION.

From the experimental results, it appears that the drug stimulates all organs having involuntary musculature, and also increases the arterial blood pressure. Amongst the organs containing involuntary musculature, those having plain muscle, like the bladder and the intestines, have their contraction diminished considerably, though not completely, after atropin. The contraction of the striated involuntary muscles of the heart are not affected by atropin, but the rhythm is accelerated. The unstriated muscles of uteri, whether of the cat or of the guinea-pig, pregnant or non-pregnant, which are stimulated by dæmia, contract less if previously treated with atropin. The contractions of the frog's arterioles, which normally follow the use of dæmia, increase further after atropin, but not after ergotoxin. It seems, therefore, that the drug exerts a direct stimulant action on involuntary muscles, and possibly also stimulates the post-ganglionic cholinergic nerves in the structures concerned. The rise of the arterial blood pressure may be accounted for by increased peripheral resistance, brought about by the contraction of the arterioles, together with the increased contraction of the heart and the larger output therefrom.

The action of the drug on the uterus appears to be of importance, inasmuch as, in the intensity of contraction produced, it compares favourably with pituitrin; and though the increased tone appears to be sustained at a lower level, it seems possible that this drug of vegetable origin may prove to be a worthy substitute for pituitrin.

SUMMARY AND CONCLUSION.

1. An active constituent of glucosidic nature has been isolated from *Dæmia extensa* in a fairly pure condition, and appears to be toxic to white mice, frogs, guinea-pigs and cats.

2. It has a stimulant action on the involuntary muscles, plain or striated, and a pronounced effect on the circulatory system, raising the arterial blood pressure appreciably.

3. Its action on the uterus appears comparable with that of pituitrin, for which it may prove to be a possible substitute.

4. Its effects appear to be due to (1) the direct stimulation of involuntary muscles, and possibly (2) the stimulation of post-ganglionic cholinergic nerves in the structures concerned.

Thanks are due to Drs. M. L. Chatterjee and B. S. Kahali, whose earlier works with the drug supplied valuable information for the present investigation. Thanks are also due to Mr. S. C. Ganguly for his valuable suggestion in the preparation of the paper. As for the assistants, who helped ungrudgingly during the course of the investigation, the authors have much pleasure in recording herewith their sense of deep appreciation of their services.

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SECTION OF THE HYPOTHALAMUS TO REMOVE THE HYPERGLYCAEMIC EFFECT OF URETHANE.*

BY

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DURING recent years a good deal of work has been done on the functions of the hypothalamus, which are now known to be numerous. It is generally believed that there are several nuclei in the hypothalamus which have been classified into three groups, namely, supra-optic, infundibular and mamillary. These nuclei give the reactions of the autonomic nervous system. Stimulation of the anterior region of the hypothalamus produces increased gastric tone and motility (Heslop, 1938), fall of blood pressure and slowing of the heart rate (Wang and Ranson, 1940), activation of the bladder (Wang and Harrison, 1939), constriction of the pupil (Hess, 1939), sleep (Hemingway *et al.*, 1940; Hess, 1932), vasodilatation, sweating and polypnoea (Beaton *et al.*, 1941; Magnon *et al.*, 1938); whereas stimulation of the posterior region results in inhibition of movement of the large bowel (Sheehan, 1942), acceleration of the heart, dilatation of the pupil (Hess, 1939), rage reactions (Fulton and Ingraham, 1929; Beattie *et al.*, 1930), rise of blood pressure and increase of blood sugar (Beattie *et al.*, 1930).

As early as in 1909 Karplus and Kriedl had shown that electrical stimulation of the hypothalamus produced excitation of the sympathetic nervous system. Beattie *et al.* (1930), Beattie and Kerr (1936) and Beattie and Sheehan (1934) proved beyond doubt that stimulation of the anterior region of the hypothalamus produced a parasympathetic response and that of the posterior region a sympathetic one. Beattie *et al.* (1930) also traced the presence of tracts of fibres from the hypothalamus to the spinal sympathetic centres.

Fulton and Ingraham (*loc. cit.*) had demonstrated that tracts of fibre arise from the frontal lobe which go to the hypothalamic nuclei. When these cortico-hypothalamic tracts were divided the hypothalamus was released from the cortical control and produced a condition of chronic rage in animals. Also in these animals there were evidences of outburst of sympathetic activities. They also showed that lesions in the pre-chiasmatic region of the hypothalamus produced changes in the behaviour of the animals, an increase in the 'wildness' was produced. Bard (1928) suggested that these subconscious manifestations were probably due to the activity of the posterior hypothalamic region. In lesions posterior to the level used by Fulton and Ingraham (*loc. cit.*) opposite effects were observed; the animals which had a tendency to be furious and wild became docile. Clark *et al.* (1939), working on cats, showed that lesions in the anterior hypothalamic region produced serious impairment of heat-dissipating reactions, whereas lesions in the posterior part of the hypothalamus caused subnormal body temperature.

Bulatao and Cannon (1925) had shown that removal of the cerebral cortex was followed by pronounced hyperglycaemia and the same effect had also been obtained by Mellanby (1919) in cats after section at the level of the superior corpora quadrigemina. On the other hand, it is also known that sections of the spinal cord at the level of the upper cervical region always produce a fall in the blood-sugar level (Table II, cat *a*). It is, therefore, noticed that sections of the brain and brain stem would produce a condition of hyperglycaemia or hypoglycaemia according as the sections passed through the fore or hinder parts of the brain. It thus stands to reason that it is essential to know the exact level through which the section will pass in order to produce the particular effect on blood sugar in experimental animals. Any section from the cerebral cortex to the colliculo-chiasmatic plane will invariably produce the condition of hyperglycaemia. But though it has been proved beyond doubt that stimulation of the posterior hypothalamic region produces hyperglycaemia yet the most anterior plane through which the section is to be made in order to abolish this hyperglycaemia effect is not definitely

* Subsequently read at the Physiology Section of the 33rd Session of the Indian Science Congress held in January 1946.

known. Brooks *et al.* (1941) in their study on hyperglycæmia produced by morphine in normal cats showed that decerebration at the mid-collicular level produced no rise of blood sugar with morphine. But these observers had not mentioned the plane of the section; their lower limit of the section had not been given. It was, therefore, decided to undertake this investigation in order to ascertain, as accurately as possible, the most anterior plane through which decerebration will have to be performed so as to abolish the hyperglycæmic effect of the hypothalamus.

Cats were used in all these acute experiments and urethane was employed as the anæsthetic unless otherwise stated. Urethane itself produced a marked and persistent rise of blood sugar in anæsthetized cats. A characteristic result is given in Table I. Decerebrations were performed at different levels and the results on the blood sugar were recorded. A typical record of the hyperglycæmic effect that was observed in cats when the plane of the section passed from the anterior border of the superior corpora quadrigemina above to the posterior border of the optic chiasma below had been shown in Table II, cat *b*. It would be noticed that there was a marked, persistent and steady rise of blood sugar. Similar results were also obtained when decerebration was done through the same plane under chloroform and ether anæsthesia. But when the plane of the section passed through the colliculo-infundibular plane, i.e. from the same point above to the anterior border of the infundibulum, the results were variable. Finally, it was observed that when the lower limit of this section was shifted further and further backwards so that it passed through or behind the mamillary bodies, i.e. decerebration was done at the colliculo-mamillary plane (Plate I), there was always a progressive fall in the blood-sugar level of the animals. It was also observed that when the plane of this section passed from the mid-collicular space above to the mamillary bodies below, either through or behind the mamillary bodies, it produced the same effect. Any of these animals that showed clotted blood at the site of the section was discarded because the irritation produced by the clot vitiated the results. Two typical results of the section at the colliculo-mamillary plane are given in Table II, cats *c* and *d*.

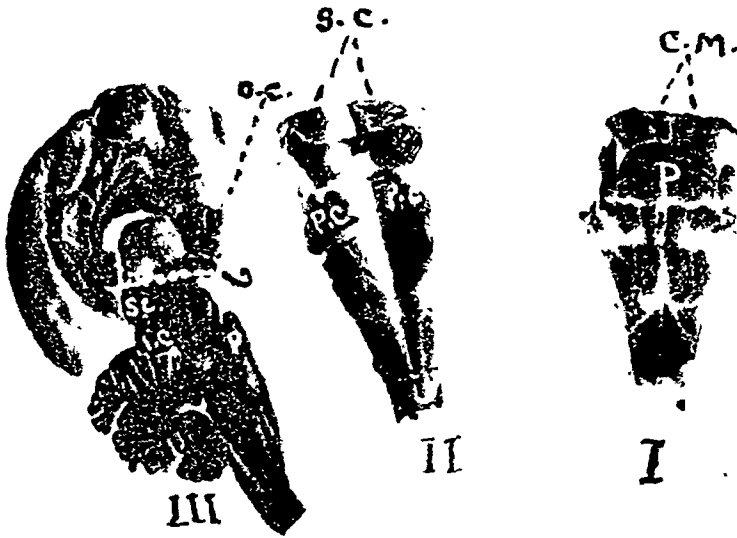
TABLE I.

Weight of cat in grammes.	Femoral vein blood sugar, in mg./per cent, in urethanized cat at intervals of 15 minutes.										
Male 2,575 ...	174	186	210	216	235	250	264	276	295	320	364

TABLE II.

Weight of cat in grammes.	Blood sugar (mg. per cent) before operation at intervals of 15 minutes. Urethane anæsthesia.	Nature of operation. Interval between last pre-operative and first post-operative samples of blood is 1½ hours.	Blood sugar (mg. per cent) after operation and post-operative rest. Intervals between succes- sive samples are 15 minutes.
(a) Male 3,000 ...	210, 220, 230, 230	Spinal preparation	235, 230, 226, 217, 188, 177.
(b) Female 2,590	165, 169	Level of decerebration—superior cor- pora quadrigemina and posterior border of optic chiasma.	228, 235, 253, 260, 267, 276, 284, 296, 305.
(c) Male 2,910 ...	199, 210, 210	Level of decerebration—superior cor- pora quadrigemina above and mamillary bodies below.	198, 190, 186, 181, 170.
(d) Male 2,900 ...	238, 243	Level of decerebration—superior cor- pora quadrigemina and mamillary bodies.	238, 232, 222, 222, 212, 202, 186.

PLATE I.



Figs. I and II.—Brain of cat, section through the colliculo-mamillary plane.

Fig. I.—Viewed from below.—

C.M.—Corpora mamillaria (cut posterior margin).

P—Pons.

Fig. II.—Viewed from above, cerebellum removed.—

S.C.—Superior corpora quadrigemina.

P.C.—Peduncles of cerebellum.

Fig. III.—A median sagittal section of the brain of cat, showing the position of the section through the colliculo-mamillary plane (a-b).

S.C.—Superior corpora quadrigemina.

I.C.—Inferior corpora quadrigemina.

O.C.—Optic chiasma.

a—Anterior border of superior corpora quadrigemina.

b—Mamillary body.

P—Pons.

a-b—Level of section through the colliculo-mamillary plane.

SUMMARY.

The most anterior plane through which section is to be made to abolish the hyperglycæmic effect of the hypothalamus passes through the anterior border of the superior corpora quadrigemina above and mamillary bodies below.

In conclusion, the author wishes to thank Lieut.-Colonel R. Linton, B.Sc., M.B., Ch.B., I.M.S., Principal, Medical College, Calcutta, for kindly giving him all facilities to complete this work. Though this work was started in 1942, for various reasons it was not possible to finish it earlier. Thanks are also due to the author's assistants for help in carrying out this work.

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THE COMPARATIVE ACTION OF SOME ALKYL HYDROCUPREIDINES.*

BY

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IN this paper the author has made a study of the comparative action of some quinine derivatives which have been formed from hydroquinidine by altering the methoxy group. Ethyl hydrocupreidine has been formed by changing the group OCH_3 into OC_2H_5 , and propyl, butyl, amyl, etc., have been obtained by substituting the higher alkyl groups. These compounds were synthesized by Ghosh and Chatterjee (1931, 1932) of the Calcutta School of Tropical Medicine and were supplied to the author.

A comparative study of the action of some similar quinine derivatives had been made by other workers, and the author, working in collaboration with the late Professor W. E. Dixon of Cambridge (Dixon and De, 1927), showed that the intensity of action varied with the change of its structure. These bases being insoluble, their hydrochlorides were used in all these experiments. The effects of hydroquinidine, ethyl, iso-propyl, iso-butyl, iso-amyl and sec-octyl hydrocupreidines have been studied.

Action on isolated intestine.—Comparative effects of different derivatives were studied on isolated pieces of intestine of kitten. Each drug was tested on several strips of the intestine in different dilutions. Hydroquinidine in dilution of 1 in 80,000 slightly increased the height of automatic movements and the tone, whereas ethyl hydrocupreidine though it reduced the tone, did not markedly reduce the automatic movements which in some cases continued for a long time. Further, it has been observed that after some time these latter movements even had a tendency to increase. With the rest of the derivatives when tested with the higher and higher members of the series in the same dilution a progressive diminution of the tone and automatic movements was noticed. With the concentration of 1 in 40,000 and above, all these compounds had a depressant action on the tone and automatic movements of the isolated intestinal loops. The intensity of action was steadily increased from ethyl hydrocupreidine as higher and higher members of the series were tried and the maximum effect was observed with iso-amyl hydrocupreidine (Graph 1, figs. *a* to *f*). The loss of tone of the intestinal muscle was very significant as it had been noticed that the tone and automatic contractions of the plain muscle of the uterus were increased. A similar increase of tone of the plain muscle of the uterus was also observed by the author while working with some other quinine derivatives in collaboration with late Professor W. E. Dixon (*loc. cit.*). Like other derivatives, especially quinidine, these substances in many instances changed the irregular and asynchronous contractions of the intestine into regular ones.

Action on the cardiovascular system.

Intact animal.—The effects of all these derivatives were tested on cats anaesthetized with chloralose. In adequate doses they showed a depressant action on the blood pressure; this effect was least with hydroquinidine; in some cases hydroquinidine produced a slight rise of blood pressure after a slight fall. Iso-butyl and iso-amyl hydrocupreidine derivatives always caused a marked fall (Graph 2, fig. *a*). The sec-octyl hydrocupreidine, in practically every case, showed a fair rise after a moderate fall (Graph 2, fig. *b*). When injections were given slowly into the femoral vein the volumes of the intestine and kidney increased in proportion to the fall of blood pressure (Graph 2, fig. *c*), and the increase in volume always preceded the fall of blood pressure thus showing that the fall is at least partly due to the

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dilatation of the vessels. An exception, however, was observed in case of sec-octyl hydrocupreidine. The kidney volume was found to be diminished with the rise of the blood pressure observed with the sec-octyl derivative (Graph 2, fig. *b*). Constriction of these vessels was possibly the cause of this rise of blood pressure. In case of pronounced rise of blood pressure the kidney and the intestinal volumes, especially the latter, showed an increase following the rise of blood pressure, signifying that this increase in volume is due to passive dilatation of vessels of these organs. A sharp and immediate fall of blood pressure was observed after rapid intravenous injection of these derivatives. A part of this effect at least must, therefore, be attributed to the depressant action of these derivatives on the myocardium.

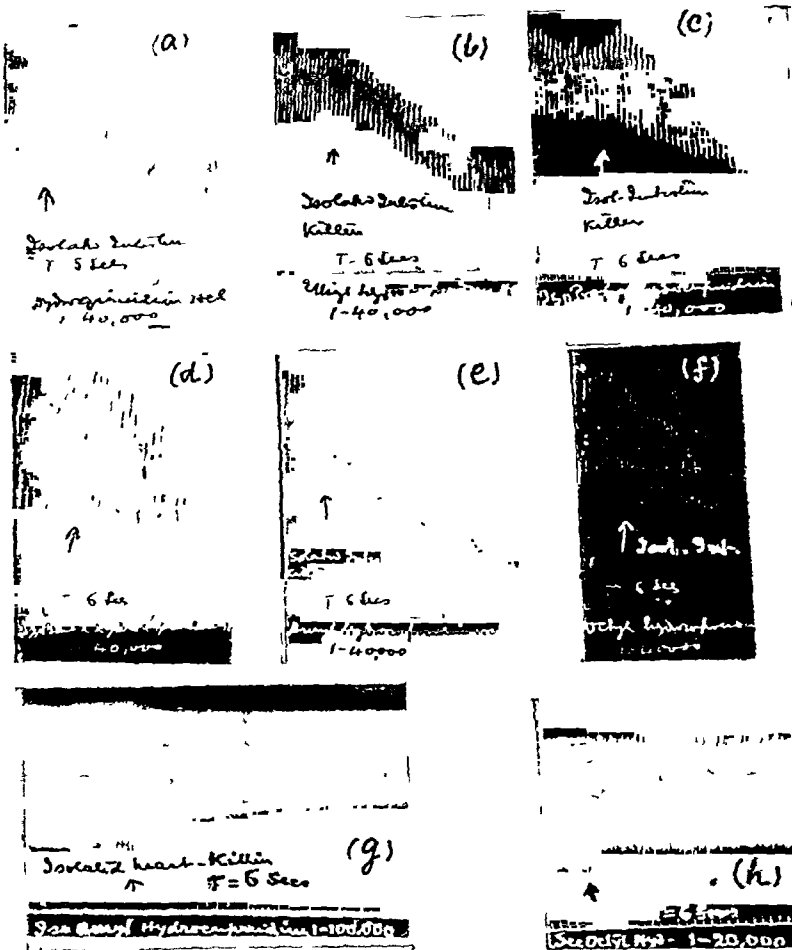
Pulmonary pressure.—Records of the pulmonary pressure were taken in cats anesthetized with chloralose, with a manometer filled with half-saturated solution of sodium sulphate. The hydroquinidine showed a slight rise followed by a fall in most instances; whereas with the higher and higher members of the series a more and more marked rise was noticed, and the subsequent fall which was observed in the case of hydroquinidine practically disappeared. Iso-butyl and iso-amyl hydrocupreidine derivatives showed a marked rise of pulmonary pressure only. The subsequent fall observed especially with hydroquinidine was probably caused by the fall of systemic blood pressure due to the dilatation of blood vessels in other parts of the body. A careful study of the tracing showed that the pulmonary pressure rose immediately and the systemic pressure fell after a few seconds. It is, therefore, evident that the drug reaching the right side of the heart first was a determining factor in the rise of the pulmonary pressure (Graph 2, fig. *a*). The high rise observed especially with iso-butyl and iso-amyl hydrocupreidine was probably due to the constriction of the pulmonary vessels. To prove this, experiments were performed in which the left auricular pressure was recorded by pushing a cannula into the left auricle through a pulmonary vein. A fall of pressure was observed. This is possible in the case of constriction of pulmonary vessels. Experiments were also performed to see how the effects of these derivatives on the pulmonary pressure compared with the effects of quinine and it was observed that the rise of the pulmonary pressure with these derivatives was always greater than that due to quinine.

Isolated heart.—The heart of kittens perfused with 1 in 50,000 strength of hydroquinidine showed in a few cases a very slight increase in amplitude, and in other cases no effect whatsoever was observed, whereas the same dilution of the other members of the series showed a diminution of the amplitude of contraction which was followed by slowing, when the perfusion was continued for some time. The maximum effect was observed in the case of iso-amyl hydrocupreidine, which showed a fair reduction of the amplitude of contraction even in 1 in 100,000 dilution (Graph 1, fig. *g*). Occasionally, the diminution in the amplitude of contraction was followed by an increase in the amplitude in cases of iso-propyl and sec-octyl hydrocupreidines in weaker solutions. With stronger solutions the heart always showed a reduction of the amplitude with every member of the series (Graph 1, fig. *h*). The coronary outflow was reduced with the reduction in amplitude.

Myocardiograph experiments.—The contractions of auricles and ventricles were recorded separately. Injections of hydroquinidine in 5-mg. doses produced a slight increase in the amplitude of contraction of both auricles and ventricles, whereas injections of similar doses of the other derivatives showed more or less reduction in the amplitude of contraction, this being especially evident in the case of iso-amyl hydrocupreidine. The sec-octyl derivative, however, with the same dose, produced dilatation of both auricles and ventricles and their force of contraction was reduced.

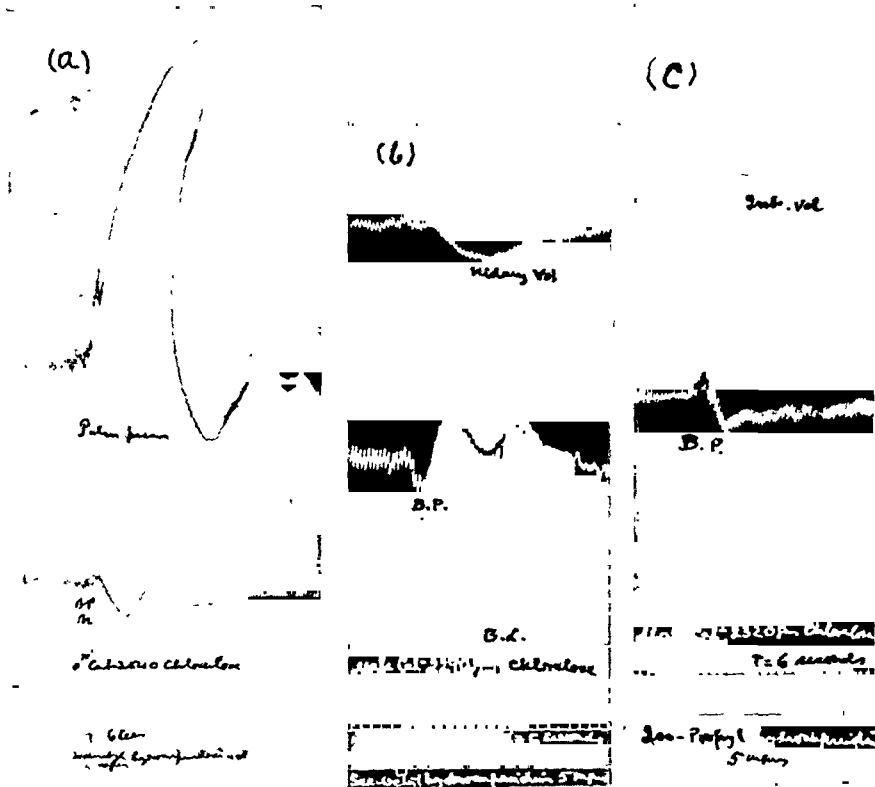
Respiratory system.—The effect of these derivatives on bronchioles was recorded on urethanized or chloralosed cats. It was observed that injections of 5 mg. of hydroquinidine or ethyl hydrocupreidine had practically no effect; while a bronchial constriction-like effect was observed with the other derivatives. This effect was very intense with iso-amyl hydrocupreidine and sec-octyl hydrocupreidine, especially when the doses were repeated. Further, it was observed that this effect was a lasting one, and was not relieved by injections of adrenalin or atropine. This effect, combined with the fact that these derivatives produce a rise of pulmonary pressure by producing constriction of the pulmonary vessels, suggests strongly that the so-called bronchial constriction-like effect is due to cedema of the lungs.

GRAPH 1.



- (a) to (f)—Show the effect of application of various quinine derivatives to isolated pieces of intestine of kitten. Strength of derivatives in solution is 1 in 40,000 in each case. (a)—Hydroquinidine, (b)—Ethyl hydrocupreidine, (c)—Iso-propyl hydrocupreidine, (d)—Iso-butyl hydrocupreidine, (e)—Iso-amyl hydrocupreidine, (f)—Sec-octyl hydrocupreidine, (g) and (h)—Perfusion of isolated hearts of kitten with Locke's solution: (g)—Shows the effect of 1 in 100,000 solution of iso-amyl hydrocupreidine, (h)—The effect of 1 in 20,000 solution of sec-octyl hydrocupreidine. Time—6 seconds.

GRAPH 2.



- (a)—Male cat, 2,540 g. chloralose. Pulmonary pressure above and carotid blood pressure below. Shows the effect of injection of 2.5 mg. of iso-butyl hydrocupreidine in the femoral vein.
Time—6 seconds.
- (b)—Male cat, 2,290 g. chloralose. Kidney volume above and carotid blood pressure below. Shows the effect of injection of 5 mg. of sec-octyl hydrocupreidine in the femoral vein.
Time—6 seconds.
- (c)—Male cat, 2,320 g. chloralose. Intestinal volume above and carotid blood pressure below. Shows the effect of injection of 5 mg. of iso-propyl hydrocupreidine in the femoral vein.
Time—6 seconds.

A similar effect had been observed by Dixon and De (*loc. cit.*), while working with some other quinine derivatives. On section, these lungs exuded a frothy fluid on pressure.

DISCUSSION.

The derivatives of this series have a depressant effect on plain muscles and produced a fall of blood pressure, due partly to the effect of dilatation of the vessels of the organs and also partly to the depressant action of the drugs on the myocardium. The force of contraction of the heart has been shown to be reduced. The rise of blood pressure observed with sec-octyl hydrocupreidine is due to vasoconstriction of the organs of the body; the kidney volume has been shown to be diminished. The dilatation of auricles and ventricles, observed with the experiments on myocardiograph with sec-octyl hydrocupreidine, is also due to this vasoconstriction.

These derivatives produced a rise of the pulmonary pressure and a fall in the left auricular pressure. These have been shown to be due to the constriction of pulmonary vessels, possibly of the venules. The constriction of the arterioles, though it will produce a rise of the pulmonary pressure, will not produce an cedema of the lungs; this effect is only possible when the pulmonary venules are constricted. A similar effect was observed by Dixon and De (*loc. cit.*) working with other quinine derivatives. Therefore, it can be taken that the pulmonary cedema is a secondary effect of the rise of the pulmonary pressure and is due to constriction of the pulmonary venules.

SUMMARY.

The intensity of action was increased from hydroquinidine and ethyl hydrocupreidine as derivatives higher and higher in the series were tested. The sec-octyl hydrocupreidine behaved a little differently. All these derivatives in adequate doses had a depressing action on the plain muscle of the intestine and the cardiac musculature. The fall of blood pressure was partly due to the depression of the myocardium and partly to the dilatation of vessels of the organs. There was a rise of pulmonary pressure, due to the constriction of pulmonary vessels. Pulmonary cedema was observed with higher members of this series.

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STUDIES ON COAGULATION OF BLOOD.

BY

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INTRODUCTION.

AN analysis of the extensive literature on the subject of coagulation of blood reveals an unusual similarity in the methods of attacking the problem. Most of the workers have attempted to separate, in a state of purity, the substances assumed to take part in the act of clotting. Using varying methods, physical and chemical, they have obtained from the delicate protein complexes concerned, fractions which in some cases have been given different names by different workers while in other instances fractions known by the same name are different in character. Howell (1935) holds the view that no satisfactory progress towards uniformity of results is to be expected until the various reagents like fibrinogen, prothrombin, etc., can be prepared in purified and standardized form. Undoubtedly, the recent work of Cohn (1945) and his associates in the fractionation of plasma proteins has yielded results of great importance, e.g. the use of gamma globulin in measles and of fibrin films in wounds. Philpot (1945) comments: 'The monograph on the proteins of blood serum by Kai O. Pederson from Prof. Svedberg's laboratory at Upsala brings home forcibly the present complexity of the subject. Although any one of the normal methods of analysis gives apparently clear-cut fractions, each fraction when analysed by some other method usually turns out to be a mixture. After a heroic attempt to achieve a complete separation, chiefly by means of ammonium sulphate with ultra-centrifugal analysis, Dr. Pederson had to confess defeat.' In addition to these difficulties, there is an inherent defect in this method of approach in that substances thus obtained after drastic and repeated chemical and physical treatment may not necessarily be the same or have the same properties as the original finely balanced complexes of the plasma and serum. A new approach to the subject, using native substances in the plasma and serum as far as possible and making a study of anti-coagulants of various types and their mode of action, was considered likely to throw some light on the matter and the following investigations were carried out along these lines.

MATERIALS AND METHODS.

The major part of the work has been carried out on blood taken from apparently normal healthy sheep. To test for the possible effects of difference in species on the mechanism of clotting, small numbers of other animals including monkey, rabbit, guinea-pig and fowl from good healthy stock have been used. Specimens of human blood have been obtained in the course of the routine work of the clinical laboratory, excluding those specially collected from patients with pneumonia and pregnancy from the local hospitals. The occasional use of oxalated blood for estimation of sedimentation rate is explained by the above.

Blood has been collected from the sheep from the jugular vein under aseptic precautions into sterile test-tubes containing the reagents in accurately measured quantities. After the observation that vigorous shaking for more than 5 minutes, even without glass-beads, tended to result in varying degrees of defibrination of blood, only the minimum of shaking required to mix the blood and the reagent has been used. Plasma has been separated by centrifuging for 15 minutes at 2,500 r.p.m. The serum collected has been used in the experiments either in its active state or inactivated at 56°C. for 30 minutes or oxalated by the addition of 0.1 c.c. of a 2 per cent solution of potassium oxalate for each cubic centimetre of serum. Blood also has been oxalated in the same proportion while it has been citrated by the addition of 0.25 c.c. of a 3.8 per cent solution of sodium citrate for each cubic centimetre of blood. So far as other reagents are concerned, stock solutions have been diluted and used and the final dilution of the reagent in the system concerned is indicated in

the text by the number following. Thus, formalin 200 means formalin 1 in 200 or 0.5 per cent, and zinc sulphate 500, a solution of zinc sulphate in the strength 1 in 500 or 0.2 per cent in the final volume of blood or plasma and reagent. To eliminate the possibility of impurities in the reagents interfering with the results, in addition to using high-grade chemicals, experiments have been repeated, using the same reagent made by different manufacturers, e.g. commercial formaldehyde by Gurr, Merck, Schering-Kahlbaum and Evans, calcium chloride by May and Baker, Merck and other firms.

Filtration of plasma and serum has been carried out in a Seitz filter, using Ford's sterimats (G. S.) grade. Brain extracts used have been obtained from fresh guinea-pig or rabbit brain, 1 g. per c.c. of normal saline.

The experiments have in general been carried out in test-tubes 100 mm. \times 12.5 mm. in size and, unless otherwise mentioned, at room temperatures, which for the particular laboratory ranged from 19°C. to 24°C. Unless otherwise stated, equal quantities of material like plasma and serum or plasma and calcium chloride solution have been used usually, in quantities of 0.5 c.c. or 1 c.c. The calcium chloride solution used to induce clotting in citrated and oxalated blood and plasma was of 0.25 M. strength, as in Quick's prothrombin time test.

The occurrence of clotting, gel-formation, etc., in the experiments were confirmed by microscopical examination of the material. This is essential as agglutination, gel-formation or coagulase production under certain circumstances are liable to be mistaken for clotting if reliance is placed on naked-eye appearances alone.

Specimens of blood from over 70 sheep, 30 human patients, 10 guinea-pigs and 6 rabbits have been used in the experiments, in addition to samples from fowl, monkey and chameleon.

Commercial formaldehyde.—During the course of experiments on erythrocyte sedimentation rates, the presence of commercial formaldehyde in small quantities was found to retard the E.S.R. and in larger amount, to inhibit it completely as shown in Table I:—

TABLE I.

Clinical condition.	E.S.R. in mm. (citrate or oxalate).		E.S.R. in mm. formalin.		Dilution of formalin.	REMARKS.
	1 hour.	2 hours.	1 hour.	2 hours.		
Normal ...	3	10	<i>Nil</i>	<i>Nil</i>	200	Formalin alone.
Tuberculosis lung ...	109	112	<i>Nil</i>	<i>Nil</i>	25	„
Malaria ...	150	167	70	127	200	„
Pneumonia ...	63	97	<i>Nil</i>	1	50	„
Pregnancy ...	56	83	<i>Nil</i>	1	100	„
Bronchitis ...	13	30	<i>Nil</i>	<i>Nil</i>	25	Formalin and oxalate.
Tropical eosinophilia ...	24	39	2	5	200	„

Further work showed that formalin possessed a powerful anti-coagulant action on the blood of various species tested, e.g. man, monkey, sheep, rabbit, guinea-pig and fowl. As commercial formaldehyde is known to contain varying amounts of formic acid and methyl alcohol, it was necessary to determine whether they influenced the reaction. Accordingly, to a number of tubes each containing 1 c.c. of sheep blood were added 0.25 c.c. respectively of normal saline as control, of methyl alcohol and formalin separately in final

dilutions of 50, 100, 200 and 400, and of formic acid in dilutions 50, 100, 500 and 1,000. Clotting took place within 10 minutes in all the tubes excepting those containing formalin, where with dilution 400, clot formed in 35 minutes and with dilutions 50, 100 and 200, no clotting occurred, even at the end of 72 hours. Crystals of sodium formate were found to be anti-coagulant but sodium formate in solution in dilutions 100 to 8,000 had no effect on clotting and in dilutions 25 and 50, clotting occurred within 10 minutes. It is evident from the above that the anti-coagulant action is exerted by formaldehyde alone.

Similar experiments using acetaldehyde, paraldehyde, acetone, ethyl alcohol and mercury perchloride in dilution 200 showed that none of them had anti-coagulant properties.

The minimum effective concentration of formalin was determined by adding to tubes with 1 c.c. of sheep blood varying dilutions of formalin, i.e. 100, 200, 250, 300 and 350. While the saline control clotted in 5 minutes, clotting took place in 10 minutes with dilution 350, in 15 minutes in dilution 300 and in 25 minutes in 250. There was no clotting with dilutions 100 and 200. A series of such experiments showed that formalin 200 prevented coagulation in the normal blood of various species. The few exceptions noted in sheep and man will be discussed later.

Commercial samples of formalin are known to be acid in reaction (pH 4 approximately). To decide whether the acidity was responsible, neutral formalin was prepared according to the method suggested by Todd and Sanford (1939). Its pH was adjusted to 7.4. Experiments using neutral formalin showed that (1) the anti-coagulant action of formalin is unaffected by adjustment of pH to neutrality, and (2) the minimum effective concentration of neutral formalin is the same as that of commercial formalin, viz. 1 in 200. Neutral formalin were subsequently used in this work.

Attempts at producing clotting in formalized blood and plasma by the addition of calcium chloride solution, serum, brain extract with or without calcium all ended in failure. Apparently, blood and plasma on treatment with formalin become permanently incoagulable. Citrated or oxalated plasma, usually liable to be clotted with such treatment, also become incoagulable in the presence of formalin. The only other anti-coagulant found to have a similar permanent action was zinc sulphate solution. The similarity between blood treated with formalin or zinc sulphate and defibrinated blood, as regards permanent incoagulability and retardation of the E.S.R., suggested that, in all these cases, incoagulability is due to the fibrinogen being either chemically altered or removed. Blood from the same animal was received into separate tubes containing formalin 100 and 200, sodium carbonate, potassium carbonate, trisodium phosphate and sodium fluoride 200, zinc sulphate 200 and 500 and the usual strength of citrate and oxalate. A saturated solution of sodium chloride was added to the plasma from these specimens of treated blood, in equal volumes. Fibrinogen was precipitated in all the tubes excepting those with formalin and zinc sulphate. Obviously, in the other tubes, the anti-coagulant action is exerted on other material, while with formalin and zinc sulphate, the action is on fibrinogen. Either the fibrinogen is in a soluble stable form or it has been precipitated earlier and carried down in the deposit in centrifuging. To decide this issue, citrated plasma in equal quantities was taken in four tubes, one kept as control and the others treated with formalin, neutral formalin and zinc sulphate solution respectively. After half an hour, sodium chloride saturated solution was added to the four tubes. Fibrinogen was precipitated in the control tube but not in the tubes with formalin, which showed slight opalescence. In the zinc sulphate tube, a slight precipitate was formed which became more marked on further addition of zinc sulphate. It would seem that zinc sulphate precipitates other protein fractions also, in addition to acting on fibrinogen and making it incoagulable. Formalin, on the contrary, keeps the fibrinogen in a soluble stable form, not precipitable on half saturation. The fact that formalin prevents coagulation by its action on fibrinogen does not necessarily mean that it has no other action on blood. Its well-known action on serum globulin is the basis of the formol-gel test and has been utilized in a quantitative estimation of globulin by viscosity measurement by Foster, Biguria and Adams (1943). The increase in viscosity of blood treated with formalin may be one of the factors in the retardation of the E.S.R., apart from the change in fibrinogen which has the same action. It will be shown later that it has probably an action on prothrombin also. Regarding its action on fibrinogen, mention was made earlier of certain exceptions

where blood from several sheep and one man clotted in spite of the use of formalin. The man was known to be suffering from a severe infection with *P. vivax* and his blood clotted within 10 minutes with formalin 200, in 6 hours with formalin 100 and in 7 hours with formalin 50. These apparently contradictory findings can be explained easily if it is assumed that formalin reacts with fibrinogen on a quantitative basis. Fibrinogen is known to be increased in a number of conditions from the normal 250 mg. to 400 mg. per cent to such high figures as 1,500 mg. per cent, for example, in pneumonia. Any such increase will require proportionately larger amounts of formalin for anti-coagulant effect. This hypothesis was put to the test by collecting specimens of blood from the local hospitals from patients with pneumonia and pregnancy, in formalin 200, 100, 50 and in citrate as control. The citrated blood did not clot while all the specimens in formalin 200 clotted within 2 hours. Those in formalin 100 did not clot with one exception, which required formalin 50 to prevent coagulation. The above findings strongly suggest that formalin reacts with fibrinogen on a quantitative basis in the reaction which prevents coagulation.

How exactly formalin acts is not clear. Quite possibly, its action on fibrinogen may be the same as that on amino-acids, as in Sørensen's formol-titration method. Mathews (1936) holds that the free amino groups in the protein molecule may be blocked by formaldehyde, so that proteolytic enzymes which normally combine with protein at this point can no longer unite with it. The detoxication of ricin, diphtheria toxin and other poisons by formaldehyde is explained by him on the basis of this mechanism.

During the course of this work, the effect of formalin on blood was also studied from other angles. Its well-known preservative action is exerted on blood as on other tissues. Formolized blood keeps well for days with very little change, in sharp contrast to oxalated blood. Thus, blood from the same rabbit was taken, treated with oxalate in one tube and formalin in another. Small quantities were kept in sealed ampoules. Total and differential leucocyte counts on the day of collection and after 5 days gave the results shown in Table II :—

TABLE II.

		FIRST DAY.		SIXTH DAY.	
		Oxalate.	Formalin.	Oxalate.	Formalin.
Total leucocytes	...	8,800	8,400	6,500	8,300
Neutrophiles	...	40	38	Cells too badly de-generated for identification.	41
Eosinophiles	...	3	2		2
Basophiles	...	Nil	Nil		Nil
Lymphocytes and monocytes		57	60		57

If neutral formalin is used, there is very little difference in staining reactions except that the leucocytes take up the stain more efficiently with Toisson's diluting fluid. Formolized blood can be used for some of the biochemical tests, e.g. serum calcium estimation by the method of Kramer and Tisdall gave the following result: Plain serum, 11.5 mg. per cent. formolized plasma, 11.4 mg. per cent. Blood-sugar estimation by Maclean's method on oxalate-fluoride blood and formol blood was found to give identical results, e.g. 82 mg. per cent, while oxalate blood in the same case after 24 hours gave 70 mg. per cent. This finding is supported by the work of Denis and Aldrich (1920) who advocated the use of formalin as a preservative for oxalated blood intended for sugar determination by the Folin-Wu method. They used one drop of formalin to 5 c.c. of blood and pointed out that larger amounts, while not necessary, did not interfere with the estimation. They found that glycolysis was

prevented by formalin even after 96 hours at 20°C. to 23°C. and that blood so treated was suitable for determination of creatinine and uric acid, but that values obtained for non-protein nitrogen and urea by urease method were invariably low. Formalin can therefore be used as a preservative for specimens of blood for certain microscopical and biochemical tests when there is likely to be delay between collection and examination.

Zinc sulphate.—The anti-coagulant action of zinc salts has been recognized as far back as 1925 by Lumiere and Couturier (quoted by Pickering, 1928). Melon (quoted by Pickering, *loc. cit.*) believed that the activities which normally lead to the production of thrombin are restrained. Experiments have already shown that zinc sulphate acts, like formalin, on fibrinogen but, unlike formalin, it seems to precipitate an altered fibrinogen. It is very powerful in action, a 1 in 1,000 dilution consistently preventing coagulation and a 1 in 2,000 dilution frequently doing so. Like formalin, it seems to have an action on other plasma proteins and it increases the viscosity of blood markedly in dilutions 200 and 500. The sedimentation rate is retarded. Another interesting observation made is that zinc sulphate 200 has the property of altering the erythrocytes so that they are not lysed on treatment with distilled water as well as with the appropriate amboceptor serum. A suspension of the treated erythrocytes in 20 or 30 times its volume of distilled water remained perfectly unchanged, even after 10 days. A slide prepared from the material and stained with Leishman's stain showed red cells with the usual outlines and colour. But for the toxicity of zinc sulphate, this property could be investigated further with a view to treatment in cases of hæmoglobinuria.

Calcium chloride.—The importance of calcium in clotting has been well recognized, though not entirely explained. The statement that calcium chloride is an anti-coagulant is therefore bound to be a surprise. In an attempt to find out how far an increase in the calcium chloride concentration hastens the onset of coagulation in plasma, it was found that after a certain level, contrary to expectation, it not only does not shorten but definitely prolongs coagulation time. The test on being carried to its logical conclusion revealed that in higher concentrations calcium chloride is an anti-coagulant. From several experiments, such as the one summarized in Table III, it was found that anhydrous calcium chloride (Merck) prevented coagulation in final concentrations of 2·5 per cent (1 in 40) and above.

TABLE III.

Sheep blood.	Calcium chloride, concn. :	Result.
1 c.c.	1 in 500	Clot within 5 minutes.
1 c.c.	1 in 200	Clot within 5 minutes.
1 c.c.	1 in 100	Clot within 30 minutes.
1 c.c.	1 in 66½	Clot within 24 hours.
1 c.c.	1 in 50	No clot.
1 c.c.	1 in 33½	No clot (5 days).
1 c.c.	1 in 16½	No clot (5 days).

The inhibiting action of calcium above a certain concentration has been noted, in the case of clotting by Quick (1935) who quotes Gordon, Whitehead and Wormald as reporting its similar action in inhibiting complement activity. Other ionized salts are known to depress the activity of complement and of coagulation. It is likely that the anti-coagulant action of magnesium sulphate, sodium sulphate, sodium chloride and ammonium sulphate in certain concentrations is of a similar nature. Dilution brings about clotting in blood or plasma treated with the salts. Blood treated with calcium chloride was therefore diluted with normal

saline and it clotted within 5 minutes, when the dilution was sufficient to bring down the level of calcium chloride to below 2 per cent. The addition of distilled water in great excess had the same result, with one difference, i.e. immediate hæmolysis took place. The whole process of clotting could be observed taking place in a transparent medium within 5 minutes or even less. Calcium chloride in higher concentrations therefore appears only to hold up the reaction that culminates in clotting, without in any way changing the components taking part in it. The neutral salts may be regarded as having a similar action. The better known property of calcium chloride as a coagulant is dealt with in the work on oxalated and citrated plasma.

Sodium taurocholate.—Miles (1945) in a report to the Medical Research Council on the E.S.R. in infective hepatitis has reported a definite inhibition of sedimentation when bile salts are added in the strength of 25 mg. per 100 c.c. Sodium taurocholate was added to blood and it was found to be a weak anti-coagulant, 1 in 100 being enough to keep the blood fluid. It had a strong tendency to produce hæmolysis. Taurocholate blood and plasma clotted in 30 minutes on dilution with an equal quantity of water, while an equal quantity of serum clotted it in 2 minutes. Its action seems to be due to its concentration, like that of calcium chloride, and it only suspends the reaction.

Potassium carbonate, sodium carbonate and sodium phosphate.—In view of the similarity between complement activity and the coagulation process with regard to calcium, it was felt that change of pH may affect the latter also. Acids and the hydroxides of sodium and potassium were found unsuitable for this study owing to their permanent fibrinolytic action. The carbonates of sodium and potassium and the phosphate of sodium were selected for trial because they have a pH over 10 without any immediate fibrinolytic action. All the three substances in dilution 200 were found to be anti-coagulant and the treated plasma had a pH between 9 and 10. The addition of 0.5 c.c. of a very dilute solution—about 0.025 per cent strength—of hydrochloric acid caused clotting in 10 minutes in the phosphate plasma and in 30 minutes in the carbonate plasma, while the controls of citrated and oxalated blood did not clot with this treatment. Serum caused clotting within 12 hours. Dilution with water had no effect but excess of normal saline induced clotting. Sodium bicarbonate and ammonium carbonate, with pH less than 10, even in dilution 100, had no anti-coagulant effect. Obviously, the carbonates of sodium and potassium and sodium phosphate inhibit the reaction of coagulation by their alkalinity. Any lowering of pH induces clotting. The carbonates seem to have a lytic effect on the platelets and white cells and, to a lesser extent, on the red cells. Examination of a blood smear, after a few hours, shows hardly any platelets, while most of the white cells are found disintegrated.

Arseno-benzol.—The anti-coagulant properties of many arseno-benzol preparations on both circulating and shed blood have been recognized and studied by several workers, e.g. Flandin and Tzanck, Lournoy, etc. (quoted by Pickering, *loc. cit.*). The view that it acts on fibrinogen put forward by Anwyl-Davies and Mellanby (1923) is apparently supported by the finding that plasma treated with arseno-benzol does not throw down the usual precipitate of fibrinogen when half saturated with sodium chloride. However, on addition of serum, it clots thus :—

1 c.c. neoarsphenamine	200 blood + sheep serum 1 c.c.	= Clot 1 hour.
.. ..	100 blood + sheep serum 1 c.c.	= No clot.
.. ..	100 blood + sheep serum 3 c.c.	= Clot 30 minutes.

This suggests that its anti-coagulant property is due to an action on prothrombin or thromboplastin. It is not merely suspending the reaction like calcium chloride and sodium taurocholate, because dilution with water does not have any effect. The action on fibrinogen, though evident, does not seem to play any part in its anti-coagulant property. Smears from treated blood show many disintegrated leucocytes and masses of platelets with indistinct outlines surrounded by a bluish stain.

Fluoride.—Sodium fluoride is a common anti-coagulant, often used with oxalate for specimens of blood for sugar determination. Fluoride is known to arrest glycolysis. Its action on clotting is stated to be due to the formation of a feebly ionized calcium salt, but addition of calcium chloride solution to fluoride plasma does not induce coagulation, while

serum does. It appears more probable that its well-known anti-enzyme action may be responsible for its anti-coagulant property.

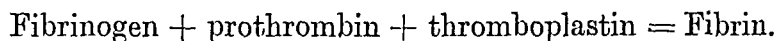
Citrate and oxalate.—Citrates and oxalates are the anti-coagulants most in use and perhaps, the most fully studied with regard to method of action. There is a large measure of agreement among workers that both of them prevent coagulation by removing the ionizable calcium, the oxalate by precipitating the insoluble calcium salt and the citrate by forming a non-ionizable calcium complex. Plasma treated with these two substances has been selected for studying the details of induced coagulation in the following experiments :—

Experiment 1.—Citrate or oxalate blood or plasma +		
calcium chloride	= Clot 6 minutes.
„ 2.—Citrate or oxalate blood or plasma +		
sheep serum	= Clot 15 minutes.
„ 3.—Citrate or oxalate blood or plasma +		
oxalated serum	= Clot 4 hours.
„ 4.—Citrate or oxalate blood or plasma +		
inactivated serum	= No clot.

Plasma and calcium chloride solution or serum have been added in equal amounts. From these results, certain provisional conclusions can be drawn. Experiment 1 shows that citrated or oxalated blood and plasma contain everything necessary for clotting excepting ionic calcium. Clotting occurs when calcium is added. Therefore, plasma contains fibrinogen, prothrombin and platelets, either entire or broken up and converted to thromboplastin. Serum also produces clotting, though after a longer interval (15 minutes) than calcium chloride solution (6 minutes). Oxalated serum also has the same effect, though with a longer interval—4 hours. Serum treated with 20 per cent oxalate solution in the same way as with 2 per cent solution gives the same result. Therefore, serum, plain or oxalated, causes clotting, through some other agency than calcium. This substance or substances are destroyed during inactivation at 56°C. for 30 minutes as seen in experiment 4. There are two possibilities: (1) pre-formed thrombin present in serum causes clotting, (2) serum contains prothrombin and formed thromboplastin. Against the first hypothesis are the following observations. Thrombin is known to disappear rapidly from serum, only 10 per cent of its original activity remaining after 20 to 30 minutes. The clotting power of serum remains unchanged for 48 hours at 20°C. to 24°C. The clotting time of plasma with fresh serum is much longer than with calcium chloride, a finding difficult to understand when the coagulation produced in a few seconds by thrombin experimentally is considered. Though some workers regard thrombin as thermolabile, thrombin prepared according to the methods of Schmidt, Howell and others is said to resist boiling at 100°C. for a short time (Pickering, *loc. cit.*), while prothrombin is generally regarded as thermolabile. The destruction of the coagulating power of serum by inactivation seen in experiment 4, does not support the thrombin theory. Serum is found to exert its action for some days and without the addition of dilute acids and alkalis required to activate the product known as metathrombin. 'Hæmolyzed' serum is unable to clot plasma, according to an experiment discussed later. From these and other considerations, thrombin does not seem to be the agent responsible for the activity of serum.

Assuming that serum contains prothrombin and thromboplastin, as distinct from platelets, it follows that plasma differs from it in some respect. It is known that plasma does not contain ionic calcium. But oxalated serum induces clotting, even though it contains only prothrombin and thromboplastin. The possibility then arises that plasma contains platelets, while any serum contains formed thromboplastin. Calcium is seen to be unnecessary in the conversion of prothrombin to thrombin and of fibrinogen to fibrin, as oxalated serum can clot oxalated blood. Since we know that calcium is necessary in clotting, we are forced to the conclusion that calcium is essential in the formation of thromboplastin from platelets. Certain experimental findings at variance with the current theory of the function of calcium in clotting support this view. Thus, Mellanby (1937) demonstrated that the venom of *L. atrox* activates prothrombin in the absence of calcium ions and that the rate of activation

is practically unaltered by the presence of potassium oxalate. The clotting produced in oxalated plasma by certain strains of *Staphylococcus aureus*; in the absence of calcium, can be explained by the usual mechanism of coagulation, provided that calcium is assumed to be necessary only in the formation of thromboplastin. The venom of *L. atrox* and certain other snakes and the material in a culture of *Staphylococcus* may be regarded as similar in structure and function to formed thromboplastin. Platelets and tissue extracts from brain, lung and testis differ from the above in that calcium is essential for the conversion of their breakdown products into thromboplastin. Certain types of snake venom such as Russell's viper venom require calcium for their activity. Ganguly (1936) has shown that viper venom acts cytolytically on the platelets of blood, enhancing their disruption; but calcium is still required for the formation of thromboplastin. Once thromboplastin is formed from platelets or tissue extracts by the action of calcium, or when an equivalent substance is supplied by the venom of *L. atrox* or by a culture of a coagulase positive strain of *Staphylococcus*, the reaction may be regarded as a simple one, thus:—



An objection to this simplification is that thrombin has not been taken into consideration. It must be pointed out that according to Pickering (*loc. cit.*) 'a satisfactory definition of thrombin is not yet possible. A pure product has not been isolated. Moreover, important chemical differences exist in the thrombins prepared by different methods and it is not known whether these differences are due solely to different degrees of purity or if bodies of different composition possess the properties of thrombin'. The material which has the power of inducing rapid coagulation of plasma has been assumed to be a single substance called thrombin. An equally satisfactory assumption would be to regard this material as a mixture of prothrombin and thromboplastin. It is noteworthy that the thrombin of Morawitz (quoted by Pickering, *loc. cit.*) is obtained by mixing tissue extract and dilute calcium chloride with serum and that of Bordet and Delange (quoted by Pickering, *loc. cit.*) by incubating the washed *débris* of blood platelets with serum and dilute calcium chloride. Other thrombins prepared by Mellanby (quoted by Pickering, *loc. cit.*) and more recently by Seegers (1940) and by Edsall, Ferry and Howard Armstrong, Jr. (1944) are all prepared by treating prothrombin with thromboplastin obtained from different tissues. It, therefore, becomes clear that the conception of thrombin as a mixture of prothrombin and thromboplastin does not seriously affect the validity of any of the proved facts in connection with the subject of coagulation.

The strength of any hypothesis is in direct proportion to its ability to explain known facts and to anticipate fresh ones. According to the reaction postulated above, plasma contains fibrinogen, prothrombin and platelets which, on addition of calcium chloride solution, break up to form thromboplastin and then cause clotting. According to this hypothesis, if platelets can be removed from plasma, re-calcification cannot cause clotting. This was put to the test by filtering plasma and serum through a Seitz filter using sterimat (G. S. grade). The results with the filtered and unfiltered reagents are given below:—

Experiment	5.—Filtered plasma	+	calcium chloride	= No clot.
"	6.— " "	+	brain extract	= No clot.
"	7.— " "	+	{ brain extract calcium chloride }	= Clot 1 hour.
"	8.— " "	+	serum unfiltered	= Clot 1 hour.
"	9.— " "	+	serum filtered	= Clot 2 hours.
"	10.—Unfiltered plasma	+	serum-filtered	= Clot 1 hour.

Experiments repeated with different specimens of blood gave similar results on several occasions. It will be seen that serum has not been affected by filtration. Plasma has undergone change. Calcium chloride solution as anticipated does not cause clotting. The platelets having been removed by filtration, there is no thromboplastin to be formed on addition of calcium. That pre-formed thromboplastin and prothrombin are not removed by filtration is proved by the finding in experiment 9. Fibrinogen is present in the filtered plasma, as shown by the clotting in experiments 7, 8 and 9, and also by the precipitation of the same on addition of a saturated solution of sodium chloride. By elimination of the various possibilities, it becomes evident that the filtration has removed the platelets only

and that the findings in the above experiments are fully in accord with those anticipated on the basis of the hypothesis suggested. Cramer and Pringle (quoted by Pickering, *loc. cit.*) passed plasma through a Berkefeld filter and obtained the same result on re-calcification as in experiment 5.

The various anti-coagulants studied can, therefore, be classified as follows :—

Treated plasma.

ADDITION OF SERUM CAUSES COAGULATION.

Coagulable by				
Permanently incoagulable.	Dilution with water	Diluted HCl	Diluted CaCl ₂	Serum only.
1. Formalin ...	1. Calcium chloride	1. Sodium carbonate	1. Oxalate	1. Neoarsphenamine.
2. Zinc sulphate	2. Sodium taurocholate	2. Potassium carbonate	2. Citrate	2. Sodium fluoride.
(Both act on fibrinogen.)	(Electrolyte concentration inhibits reaction by prothrombin. Neutral salts probably have same action.)	3. Sodium phosphate (Alkalinity suspends reaction.)	(Ca removed)	(Exact action not known. Probably acts chemically on prothrombin.)

Prothrombin and complement.—Some of these experiments have shown that complement activity and the coagulation reaction have features in common, e.g. both are inhibited by calcium in high concentration, by a pH over 9, by heat inactivation at 56°C. and so on. Many workers like Bordet, Hirshfeld and Klinger, Fuchs, Hartmann and Falkenhausen (quoted by Quick, *loc. cit.*) have been struck by the similarity between complement and prothrombin. Fuchs holds that prothrombin is identical with the complement or at least with its mid-piece. Quick concludes from a series of experiments on the subject that complement and prothrombin are not identical but appear to have some similarity in structure. Ecker and Pillemer (1941) hold that there is no correlation between the inactivation of complement and the inhibition of coagulation. Experiments undertaken to test these conflicting views proved that many of the anti-coagulants like formalin and zinc sulphate are anti-complementary in very high dilutions while others like calcium chloride and sodium fluoride are so in the concentrations used as anti-coagulants. The following experiment was carried out to obtain more direct evidence: To two test-tubes, each containing 0.5 c.c. of inactivated rabbit amboceptor serum, was added 5 c.c. of sheep's blood. The amboceptor used was theoretically in excess for the antigen. One tube was left in the incubator at 37°C. for 24 hours and the other left at room temperature. Haemolysis took place in both the tubes. The serum from the tube incubated was added to citrate or oxalate plasma. It did not produce coagulation while normal serum from the same sheep did so. The serum from the tube left at room temperature also did not cause clotting, even after 24 hours. But in this plasma, a faint film of fibrin was seen. Haemolysis by distilled water does not prevent coagulation of blood by suitable treatment with serum, CaCl₂, etc. The only substance that can be removed from the sheep blood and rabbit serum in the course of haemolysis, other than antigen and antibody, is complement. As the sheep cells and inactivated rabbit serum have nothing to do with coagulation, the obvious inference is that the serum lost its ability to coagulate plasma, because the complement has been used up. If this is correct, prothrombin and complement are either identical or possess a common component like the mid-piece.

On linking up the conclusions arrived at earlier in the paper, it is possible to obtain a fairly complete picture of the processes involved in coagulation. Blood remains fluid in the circulation because there is no formed thromboplastin to initiate the reaction. Once it escapes from the blood vessels, it comes in contact with the tissues inside the body or with substances outside the body. The platelets undergo disintegration on the water-wettable surfaces and form thromboplastin with the help of calcium. Wolpers and Ruska (1939) describe the platelets breaking up in coagulation, the protoplasmic part dissolving by intake of fluid and the granular parts acting as centres of attraction for fibrin deposition. Inside the vessels, injury to the vessel wall causing a rough surface, or the presence of any foreign body, whether inert like a thread or ligature, or active like venoms or tissue extracts, can induce intravascular clotting. The inert substances are covered with disintegrating platelets and thromboplastin while among the active foreign bodies, tissue extracts and some venoms can act only with the aid of calcium. Some, like the venom of *L. atrox* and perhaps other preparations not yet tested, behave like thromboplastin, without the aid of calcium. Once thromboplastin or an equivalent substance is formed, it combines with prothrombin and fibrinogen to form fibrin. Thrombin may be regarded as any material which contains prothrombin and thromboplastin together. The reaction may be retarded or inhibited by a number of methods, e.g. high electrolyte concentration, pH above 10, presence of certain chemical substances, etc. It may be prevented by chemical action on one or other of the reagents taking part in it, with permanent or temporary effect, e.g. formalin or citrate. The use of the terms anti-thrombin and anti-prothrombin in the sense of specific substances appears unnecessary in the face of these observations. The necessity for such terms can be traced to the idea that any intravascular clotting is progressive and will end in wholesale coagulation in several vessels. This assumption does not appear justified. It appears more logical to regard intravascular coagulation as a phenomenon of frequent occurrence in normal life, limited in amount by the exhaustion of available thromboplastin and circumscribed to areas where it is necessary, e.g. repair of damage in capillaries, arrest of bacterial movement and, perhaps, neutralization of venom and toxins. The important work of Taylor, Mullick and Ahuja (1935) on the action of viper venom has shown that, with high dosage, death occurs in a few minutes associated with extensive intravascular clotting. If death is delayed, as with low dosage, the blood remains fluid and incoagulable. They conclude that slower action produces invisible coagulation, perhaps with fine deposition of fibrin in the walls of the blood vessels and that the blood, being defibrinated, is incoagulable. This is fully in accord with the view that coagulation is a defensive mechanism of great importance, constantly in use in the system in reactions which do not cause large-scale clotting and is not merely a costly method to prevent loss of blood in a rare emergency. Its common function seems to be to deal with the presence of any foreign body entering the blood stream and to prevent it from circulating.

The probable identity or similarity between prothrombin and complement has an added significance in view of this concept. Pickering (*loc. cit.*) in a study of the embryological development of plasma proteins in the chick, found that complement could not be detected before the 13th day of development and on that day, only in those which had developed fibrinogen and prothrombin. He found that shed blood may clot on the 14th day. This developmental association between prothrombin and complement and the evidence presented earlier in support of their similarity suggest a new outlook. Coagulation may be regarded as the earliest immunity reaction in development. The antigen is supplied by thromboplastin or an equivalent material, either formed from tissue extracts or other substances with the aid of calcium or existing ready made as an antigenic complex in material like the venom of *L. atrox* or a culture of *Staphylococcus*. The antibody is fibrinogen. The specificity of action proved in the formation of fibrin in clotting and the increase of fibrinogen in response to infection or otherwise are both features satisfying the definition of an antibody. The complement is supplied by the prothrombin, an idea which does not pronounce any judgment on the vexed question of their identity but which is supported by their accepted similarity of structure. Closer examination of this new conception of coagulation as an immunity reaction reveals many established findings lending it further support.

SUMMARY.

1. Certain substances in common use have been found to possess marked anti-coagulant properties.
2. The mode of action of these substances and of some of the well-known anti-coagulants has been studied and classified.
3. A suggestion is put forward that coagulation of blood may be an immunological reaction, with fibrinogen as antibody, prothrombin as complement and any foreign body in association with thromboplastin as antigen.

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STUDIES ON THE CULTIVATION OF THE ÆTIOLOGICAL AGENT OF RABIES *IN VITRO* AND ITS NATURE.

BY

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INTRODUCTION.

NOGUCHI (1913) reported that he had succeeded in cultivating rabies virus in a medium similar to that employed by him for the cultivation of the spirochætæ of relapsing fever. Levaditi (1914) found that infected spinal ganglia kept in plasma at 37°C. remained infective for 53 days. Successful cultivation of rabies street virus in Borel cultures was reported by Stoel (1930). He used the whole head or brain of chick embryos and carried the virus through five sub-cultures during a period of 25 days. Waldheker (1935) reported wholly negative results with two different strains of fixed virus and two strains of street virus. Different media, including the Maitland medium, were tried by him. Kanajawa (1936, 1937) reported the successful cultivation of rabies fixed virus in a medium containing rabbit-embryo brain and Tyrode solution. He found that with the above medium there was a loss of virus after the tenth passage. Webster and Clow (1936, 1937) reported the propagation of rabies virus in a medium containing mouse-embryo brain and monkey serum-Tyrode. They found that rabbit- or chick-embryo brain could be substituted for the mouse-embryo brain in the medium. If serum was omitted from the medium it was not adequate to propagate a newly introduced mouse-brain virus, nor an already established culture virus. The replacement of monkey serum by horse serum in the medium, however, was without any apparent effect on the culture virus. Bernkopf and Kligler (1937) reported the successful cultivation of rabies fixed virus in a medium consisting of rat- or mouse-embryo brain and chicken plasma covered with Tyrode-serum. With the medium described by Webster and Clow (*loc. cit.*) they found that no growth was obtained with rabbit serum, but successful serial passages were possible with media containing either monkey or human serum. The virus cultures incubated at 30°C. or 37°C. were found to increase in virulence up to about the sixth or seventh day. Thereafter, the virulence declined and disappeared about the twentieth day. Parker, Raymond and Hollender (1945) propagated the rabies virus for as long as 57 passages in cultures of chopped mouse-embryo brain suspended in rabbit serum and buffered salt solutions. They found that attempts to cultivate the rabies virus in chick-embryo brain cultures were unsuccessful even when the virus had been adapted to chick tissue by serial intracerebral passage in embryonated eggs.

During the period 1937-39 attempts were made by the author to propagate rabies virus on the lines described by Webster and Clow (*loc. cit.*) and Bernkopf and Kligler (*loc. cit.*). With the media containing infected chick-embryo brain and fowl plasma covered with Tyrode-serum it was found that undiluted ground-up cultures before incubation failed to infect animals, while cultures, after incubation for 4 days at 37°C. or after serial passages, proved to be infective indicating that the virus had multiplied in the medium. It was possible to maintain the virus in plasma cultures during a maximum period of 12 days using rabbit serum. With human serum it was possible to carry the virus through six sub-cultures during a period of 23 days, suggesting that human serum was better than rabbit serum. No evidence of multiplication of rabies virus was found in a medium containing chick-embryo brain, rabbit serum and Tyrode solution. When rabbit serum was replaced by human serum in the medium there was no evident multiplication of the virus. With the addition of chick-embryo extract to the above medium, it was possible to carry the virus through twelve sub-cultures during a period of 34 days. The concentration of the virus obtained in cultures using the methods described above was, however, very low compared with the titres obtained in the brains of sheep infected with rabies fixed virus used in the manufacture of 5 per cent phenol vaccine and, therefore, was unsuited for immunization against rabies. Webster and Casals (1941), after a considerable amount of

work on culture virus, also came to the conclusion that, from a practical point of view, culture virus was not a satisfactory source of rabies vaccine due chiefly to its low content of virus.

Veeraraghavan (1945) reported that there was evidence of multiplication of rabies virus in a liquid medium containing sheep-brain extract, sheep serum and fresh young guinea-pig brain tissue. The concentration of the virus obtained in cultures, however, was not satisfactory from the point of view of preparation of a vaccine. Several attempts were made to improve the medium and it has been found that the addition of glycine and peptone to the medium considerably increased the concentration of the virus in cultures. A medium has now been evolved in which it is possible to obtain at least five times the concentration of virus normally present in the brains of infected sheep used in the preparation of antirabic vaccine.

Veeraraghavan (1944) described a protozoal parasite of the central nervous system in animals suffering from rabies, and discussed the possibility that the parasite might be concerned with the ætiology of rabies. Subsequent work (Veeraraghavan, 1945) showed that forms resembling this parasite could be encountered in *in vitro* cultures of street and fixed virus grown in media containing sheep-brain extract, sheep serum and fresh young guinea-pig brain tissue. Evidence is presented in this paper to show that the ætiological agent of rabies can successfully grow and multiply in a cell-free medium devoid of any live embryonic tissue proving that it is not a virus in the fully accepted sense of the word.

MATERIALS AND METHODS.

The method of cultivation of the virus *in vitro* was on the lines described by Veeraraghavan (1945). The particulars regarding the various constituents used in the preparation of the culture media and the technique adopted are given below:—

Brain extract.—Normal sheep's brain was dissected out with aseptic precautions from the heads of freshly slaughtered sheep obtained from the local slaughter-house. A portion of the brain was weighed and washed repeatedly in sterile distilled water to get rid of blood. It was then ground up in a mortar with sterile glass-powder under aseptic conditions, and grinding was continued until a smooth paste was obtained which, when examined under the microscope, was homogeneous and failed to show the presence of intact cells. Ice-cold, sterile distilled water was added gradually in small quantities and grinding continued until a 2 per cent emulsion was obtained. This was allowed to stand for a few minutes to permit the glass-powder to settle, after which the supernatant was centrifuged for two hours at moderate speed. The supernatant was transferred to a sterile flask, steamed in a steam-sterilizer for half an hour on each of three consecutive days, and subsequently tested for aerobic and anaerobic sterility. The 2 per cent extract was generally diluted to 1 per cent with sterile distilled water at the time of preparation of the medium. In a later part of the work concentrated brain extracts up to 10 per cent were prepared according to the above method and used in the preparation of the culture media. It was found that satisfactory results could be obtained with brain extract kept for two or three weeks in cold storage.

Serum.—Sterile sheep serum in varying concentrations was used.

Peptone.—A solution of 1·5 per cent of Difco Bacto-Peptone in distilled water was filtered through filter-paper and sterilized by steaming for half an hour each day for three consecutive days. The solution could be sterilized by autoclaving. One part of this solution was added to nine parts of the medium.

Glycine.—Varying concentrations of glycine (B.D.H.) were tried. Solutions ranging from 1·5 per cent to 20 per cent were prepared and sterilized by steaming for half an hour each day for three consecutive days.

Fresh nerve tissue.—Just before inoculating the medium with the virus, fresh nerve tissue was obtained from the brain of a young guinea-pig, one or two days old. Each brain was emulsified in about 5 ml. of distilled water and added to 45 ml. of the medium.

Virus inoculum.—A 20 per cent suspension of the virus was generally used for inoculating the culture media. The virus suspension was prepared as follows: The brain of a sheep completely paralysed after subdural inoculation with the Paris strain of rabies fixed

virus was dissected out aseptically. A portion of the infected brain was weighed. It was placed in a sterile mortar, washed repeatedly with sterile distilled water to get rid of blood, and ground into a smooth paste with sterile glass-powder. When the cell structure was found to be completely crushed on examination under the microscope it was diluted with sterile distilled water to make a 20 per cent suspension. The suspension was centrifuged for half an hour at 3,000 r.p.m. The supernatant was pipetted out and used as the inoculum.

Experimental animals.—It would have perhaps been more satisfactory to use mice for this work, for then it would have been possible to compare the results obtained with those reported by others. But, mice were not available and young healthy guinea-pigs of 250 g. to 300 g. weight were used instead.

Virus titration.—The virus content of a culture was determined by inoculating guinea-pigs of 250 g. to 300 g. weight subdurally with 0.2 ml. of various dilutions of the culture in distilled water. The highest dilution which proved infective to at least 50 per cent of the inoculated guinea-pigs was determined. From this the virus content of the culture in terms of the minimum subdural lethal dose (m.l.d.) per ml. was calculated by multiplying the highest infective dilution by five, since 0.2 ml. is the inoculum.

Sterility.—Sterile technique was used throughout and the cultures after incubation were regularly tested for sterility aerobically and anaerobically.

EXPERIMENTAL.

1. *Effect of increasing the virus content of the inoculum used in cultures.*

Veeraraghavan (1945) reported the evidence of multiplication of rabies fixed virus in a medium containing six parts of one per cent sheep-brain extract, one part of sheep serum, two parts of distilled water and fresh young guinea-pig nerve tissue. To this medium one part of the supernatant from a centrifugalized suspension of one per cent fixed virus brain was added. Inoculated subdurally into guinea-pigs in 0.2 ml. amounts the culture was infective in a dilution of 1 in 10, whereas after incubation for 12 hours at 37°C. it was infective in a dilution of 1 in 80. As the diluted culture at the start of the experiment represented a 1 in 1,000 dilution of the virus, the twelfth-hour culture was infective in a dilution of 1 in 80,000 theoretically. But, the actual virus content of the culture was only 400 m.l.d. which is a poor concentration. Attempts were, therefore, made to increase the concentration of the virus in cultures by increasing the virus content of the inoculum.

Experiment A.—Ninety ml. of the medium consisting of 60 ml. of 1 per cent sheep-brain extract, 10 ml. of sheep serum, 20 ml. of distilled water and an emulsion of the brains of two young guinea-pigs in 10 ml. of distilled water, were put into a sterile 250 ml. Erlenmeyer flask. The medium was inoculated with 10 ml. of the supernatant after centrifugalization of a 10 per cent suspension of fixed virus rabbit brain. The virus content of the flask was titrated in guinea-pigs. The flask was incubated at 37°C. for 12 hours. The twelfth-hour culture was centrifuged slowly for 10 minutes to deposit the added guinea-pig nerve tissue. The supernatant was inoculated in various dilutions into guinea-pigs. The results are given in Table I:—

TABLE I.

Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.				
	1/100	1/200	1/400	1/800	1/1,000
0	6/9*, 7/9	S, S	S, S	—	—
12	—	—	6/8, 7/9	7/9, 8/10	S, S

Note.—6/9* = Guinea-pig paralysed on the sixth day and died on the ninth day.

S = Guinea-pig remained well.

— = Dilution not tested.

Important: The above footnote holds good for all the other tables in this paper.

Comment.—It will be seen from Table I that by increasing the virus content of the inoculum there occurred a corresponding increase in the virus content of the resultant growth.

Experiment B.—The above experiment was repeated with a 20 per cent virus suspension as the inoculum. Forty-five ml. of the medium containing 6 parts of 1 per cent sheep-brain extract, 1 part of sheep serum, 2 parts of distilled water and the emulsion of the brain of a young guinea-pig, were kept in a 100 ml. Erlenmeyer flask. Nine ml. of the supernatant from a 20 per cent suspension of fixed virus sheep brain were inoculated into the medium. The virus content of the flask before and after incubation at 37°C. for 12 hours was titrated in guinea-pigs. The results are given in Table II :—

TABLE II.

Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.					
	1/500	1/1,000	1/2,500	1/5,000	1/7,500	1/10,000
0	S, S	S, S	S, S	—	—	—
12	—	—	8/10, 8/10	10/11, 10/12	S, S	S, S

Comment.—It will be seen from Table II that the culture before incubation was not infective in a dilution of 1 in 500, while the twelfth-hour culture was infective in a dilution of 1 in 5,000, indicating that the virus content was 25,000 m.l.d. per ml. By the addition of 9 ml. of a 20 per cent suspension of fixed virus sheep brain to 45 ml. of the medium the flask contained at the outset 3·66 per cent of sheep-brain virus. This percentage of virus inoculum seemed to give better results than the 10 per cent inoculum used in the previous experiment.

2. Effect of addition of peptone to the culture medium.

The effect of adding peptone to the medium was investigated.

Experiment A.—Flask A contained 30 ml. of 1 per cent sheep-brain extract, 5 ml. of sheep serum and 10 ml. of distilled water. Flask B contained 30 ml. of brain extract, 5 ml. of sheep serum, 5 ml. of 1·5 per cent peptone solution and 5 ml. of distilled water. An emulsion of the brain of one young guinea-pig was added to each flask. Nine ml. of the supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain were added to the 45 ml. of medium in each flask. In order to determine the initial concentration of the virus in the flasks, the virus content of the inoculum after dilution 1 in 6 in distilled water was titrated in guinea-pigs. After incubation for 12 hours at 37°C., the contents of the flasks were centrifuged separately for 10 minutes to get rid of the guinea-pig brain tissue and the virus content of the supernatants from each flask titrated in animals. The results are given in Table III :—

TABLE III.

	Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.					
		1/500	1/1,000	1/2,500	1/5,000	1/7,500	1/10,000
Virus inoculum diluted 1 in 6 with water.	...	S, S	S, S	S, S	—	—	—
Flask A (without peptone).	12	—	—	7/9, 7/9	8/10, S	13/15, S	S, S
Flask B (with peptone).	12	—	—	7/9, 7/9	7/9, S	7/9, 7/9	7/9, 8/10

Comment.—It will be seen from Table III that the culture of the virus in a medium containing sheep-brain extract, sheep serum and young guinea-pig nerve tissue contained 37,500 m.l.d. of virus per ml. With the addition of peptone to the medium in a concentration of 0·15 per cent, the virus content of the culture increased to at least 50,000 m.l.d. per ml. The results indicated that the use of peptone in the medium was of definite value in stimulating the growth of virus in cultures.

Experiment B.—The experiment was repeated using the same concentration of peptone in a medium containing 1·5 per cent sheep-brain extract, 2 per cent sheep serum and 2·5 per cent glycine.

Each of the flasks A and B contained 30 ml. of 10 per cent sheep-brain extract, 4 ml. of sheep serum and 25 ml. of 20 per cent glycine. To flask A were added 20 ml. of 1·5 per cent peptone and 101 ml. of distilled water. To flask B were added 121 ml. of distilled water only. To each of the flasks were added 36 ml. of the supernatant from a 20 per cent suspension of fixed virus sheep brain and the flasks were incubated under strict anærobic conditions. The virus content of the inoculum diluted 1 in 6 with distilled water and that of the cultures in flasks A and B after 24 hours' incubation at 37°C. were titrated in guinea-pigs. The results are given in Table IV :—

TABLE IV.

	Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.					
		1/2,000	1/2,666	1/3,333	1/200,000	1/300,000	1/500,000
Virus inoculum diluted 1 in 6 with water.	...	6/8, 7/9	S, S	S, S	—	—	—
Flask A (with peptone).	24	—	—	—	—	6/8, 7/9	6/8, 6/8
Flask B (without peptone).	24	—	—	—	S, S	S, S	S, S

Comment.—It will be seen from Table IV that the initial virus content of the flasks was 10,000 m.l.d. per ml. The virus content of the culture in flask A containing peptone was 2,500,000 m.l.d., while that in flask B without peptone was less than 1,000,000 m.l.d. per ml. These results confirmed the previous findings that addition of peptone was favourable for the multiplication of the virus. If the culture virus was to be used as the source of anti-rabic vaccine it was not considered advisable to increase the concentration of peptone in the medium and higher concentrations were therefore not tried.

3. *Effect of addition of glycine to the culture medium.*

Attempts made in the past (Veeraraghavan, 1941) to eliminate excess nerve tissue in antirabic vaccine by means of iso-electric precipitation of virus suspensions showed that the addition of amino-acid glycine to the buffer solution had a definite protective action on the virus. It was, therefore, considered advisable to try the effect of addition of glycine as an enrichment factor in the medium. Concentrations ranging from 0·15 to 5 per cent were tried with the following results. The exact details of the technique used are given below :—

Experiment A.—Flask A contained 30 ml. of 1 per cent sheep-brain extract, 5 ml. of sheep serum and 10 ml. of distilled water. Flask B contained 30 ml. of 1 per cent sheep-brain extract, 5 ml. of sheep serum, 5 ml. of 1·5 per cent glycine and 5 ml. of distilled water. An emulsion of the brain of one young guinea-pig was added to each flask. Nine ml. of the supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain were added to 45 ml. of the medium in each flask. The initial concentration of the virus in the flasks at the start of the experiment was determined by titrating the virus content of the

Comment.—It will be seen from Table I that by increasing the virus content of the inoculum there occurred a corresponding increase in the virus content of the resultant growth.

Experiment B.—The above experiment was repeated with a 20 per cent virus suspension as the inoculum. Forty-five ml. of the medium containing 6 parts of 1 per cent sheep-brain extract, 1 part of sheep serum, 2 parts of distilled water and the emulsion of the brain of a young guinea-pig, were kept in a 100 ml. Erlenmeyer flask. Nine ml. of the supernatant from a 20 per cent suspension of fixed virus sheep brain were inoculated into the medium. The virus content of the flask before and after incubation at 37°C. for 12 hours was titrated in guinea-pigs. The results are given in Table II:—

TABLE II.

Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.					
	1/500	1/1,000	1/2,500	1/5,000	1/7,500	1/10,000
0	S, S	S, S	S, S	—	—	—
12	—	—	8/10, 8/10	10/11, 10/12	S, S	S, S

Comment.—It will be seen from Table II that the culture before incubation was not infective in a dilution of 1 in 500, while the twelfth-hour culture was infective in a dilution of 1 in 5,000, indicating that the virus content was 25,000 m.l.d. per ml. By the addition of 9 ml. of a 20 per cent suspension of fixed virus sheep brain to 45 ml. of the medium the flask contained at the outset 3.66 per cent of sheep-brain virus. This percentage of virus inoculum seemed to give better results than the 10 per cent inoculum used in the previous experiment.

2. Effect of addition of peptone to the culture medium.

The effect of adding peptone to the medium was investigated.

Experiment A.—Flask A contained 30 ml. of 1 per cent sheep-brain extract, 5 ml. of sheep serum and 10 ml. of distilled water. Flask B contained 30 ml. of brain extract, 5 ml. of sheep serum, 5 ml. of 1.5 per cent peptone solution and 5 ml. of distilled water. An emulsion of the brain of one young guinea-pig was added to each flask. Nine ml. of the supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain were added to the 45 ml. of medium in each flask. In order to determine the initial concentration of the virus in the flasks, the virus content of the inoculum after dilution 1 in 6 in distilled water was titrated in guinea-pigs. After incubation for 12 hours at 37°C., the contents of the flasks were centrifuged separately for 10 minutes to get rid of the guinea-pig brain tissue and the virus content of the supernatants from each flask titrated in animals. The results are given in Table III:—

TABLE III.

	Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.					
		1/500	1/1,000	1/2,500	1/5,000	1/7,500	1/10,000
Virus inoculum diluted 1 in 6 with water.	...	S, S	S, S	S, S	—	—	—
Flask A (without peptone).	12	—	—	7/9, 7/9	8/10, S	13/15, S	S, S
Flask B (with peptone).	12	—	—	7/9, 7/9	7/9, S	7/9, 7/9	7/9, 8/10

Experiment C.—As glycine appeared to be beneficial to the growth of the virus the effect of higher concentrations of glycine in the medium from 1 to 5 per cent was investigated.

Each of the flasks A, B and C contained 15 ml. of 2 per cent sheep-brain extract, 0·5 ml. of sheep serum and 5 ml. of 1·5 per cent peptone. Five ml. of a 10 per cent solution of glycine and 19·5 ml. of distilled water were added to flask A. To flask B were added 6·25 ml. of 20 per cent glycine and 18·25 ml. of distilled water, and to flask C 12·5 ml. of 20 per cent glycine and 12 ml. of distilled water. An emulsion of the brain of one young guinea-pig was added to each flask. Nine ml. of the supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain were added to the 45 ml. of the medium in each flask. In order to determine the initial concentration of the virus in the flasks, the virus content of the inoculum after dilution 1 in 6 in distilled water was titrated in guinea-pigs. After incubation for 12 hours at 37°C., the contents of the flasks were centrifuged separately for 10 minutes to get rid of the added guinea-pig brain tissue and the virus content of the supernatant from each flask titrated in animals. The results are given in Table VII :—

TABLE VII.

		FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.					
Number of hours at 37°C.		1/2,000	1/2,666	1/3,330	1/30,000	1/45,000	1/60,000
Virus inoculum diluted 1 in 6 with water.	...	6/7, 6/8	9/10, S	S, S	—	—	—
Flask A (with 1% glycine).	12	—	—	—	8/11, S	S, S	S, S
Flask B (with 2·5% glycine).	12	—	—	—	6/8, 7/8	6/8, 8/10	6/8, S
Flask C (with 5% glycine).	12	—	—	—	6/8, 7/9	6/8, 7/9	S, S

Comment.—It will be seen from Table VII that the initial virus content of the flasks before incubation was 13,330 m.l.d. per ml. The virus content of flask A containing 1 per cent glycine was 150,000 m.l.d. per ml. The virus content of flask B containing 2·5 per cent glycine was 300,000 m.l.d., whereas that of flask C containing 5 per cent glycine was only 225,000 m.l.d. per ml. From this it would appear that the optimum concentration of glycine in the medium necessary for the multiplication of the virus is 2·5 per cent.

4. *Effect of altering serum content.*

In the earlier experiments the serum content of the medium was kept constant at 10 per cent. If the culture virus is to be used in the preparation of antirabic vaccine it is desirable that the serum content should be reduced to a minimum. Therefore, experiments were undertaken to determine the minimum quantity of serum necessary for the multiplication of the virus *in vitro* :—

Experiment A.—In this experiment the other constituents of the medium, viz., sheep-brain extract, glycine and peptone, were kept constant but the serum content was altered. Flask A contained 30 ml. of one per cent sheep-brain extract, 5 ml. each of 1·5 per cent glycine and peptone, and 5 ml. of distilled water. Flask B contained the same quantities of sheep-brain extract, glycine and peptone with 2·5 ml. of distilled water and 2·5 ml. of sheep serum. Flask C contained in addition to other constituents 5 ml. of sheep serum without distilled water. The emulsion of the brain of a young guinea-pig and 9 ml. of the supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain were added to each flask. The virus content of the inoculum diluted 1 in 6 and the virus content of the

inoculum diluted 1 in 6 with distilled water. After incubation for 12 hours at 37°C., the virus content of the supernatants from each flask was titrated in guinea-pigs. The results are given in Table V:—

TABLE V.

	Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.					
		1/500	1/1,000	1/2,500	1/5,000	1/7,500	1/10,000
Virus inoculum diluted 1 in 6 with water.	...	S, S	S, S	S, S	—	—	—
Flask A (without glycine).	12	—	—	7/9, 7/9	8/10, S	13/15, S	S, S
Flask B (with 0.15% glycine).	12	—	—	7/9, 7/9	7/9, 7/9	8/10, S	10/11, 12/13

Comment.—It will be seen from Table V that after 12 hours' incubation at 37°C., the culture without the addition of glycine was infective in a dilution of 1 in 7,500, whereas the culture with 0.15 per cent glycine was infective in a minimum dilution of 1 in 10,000 indicating that glycine had some beneficial effect on the multiplication of the virus.

Experiment B.—In this experiment the effect of concentrations of glycine ranging from 0.15 to 1 per cent was studied.

Each of the flasks A, B, C and D contained 30 ml. of 1 per cent sheep-brain extract, 0.5 ml. of sheep serum, 5 ml. of 1.5 per cent peptone and 4.5 ml. of distilled water. Into the flasks A, B, C and D 5 ml. of 1.5, 3, 5 and 10 per cent glycine respectively were added. An emulsion of the brain of one young guinea-pig was added to each flask. Nine ml. of the supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain were added to 45 ml. of the medium in each flask. In order to determine the initial concentration of the virus in the flasks, the virus content of the inoculum after dilution 1 in 6 in distilled water was titrated in guinea-pigs. After incubation for 12 hours at 37°C. the virus content of the supernatant from each flask was titrated in animals. The results are given in Table VI:—

TABLE VI.

	Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.				
		1/2,000	1/2,666	1/15,000	1/20,000	1/30,000
Virus inoculum diluted 1 in 6 with water.	...	6/8, 9/11	S, S	—	—	—
Flask A (with 0.15% glycine).	12	—	—	6/8, 6/8	S, S	—
Flask B (with 0.3% glycine).	12	—	—	6/8, 6/8	8/10, S	S, S
Flask C (with 0.5% glycine).	12	—	—	—	7/9, S	7/8, S
Flask D (with 1.0% glycine).	12	—	—	—	6/9, 7/9	6/8, S

Comment.—It will be seen from Table VI that the culture of the virus in a medium containing 0.15 per cent glycine contained 75,000 m.l.d. of virus. When the concentration of glycine in the medium was increased to 0.3, 0.5 and 1.0 per cent the virus content of the cultures was 100,000, 150,000 and 150,000 m.l.d. respectively, indicating that by increasing the concentration of glycine in the medium it is possible to increase the virus content of the twelfth-hour cultures.

Comment.—It will be seen from Table IX that the initial virus content of the flasks was less than 5,000 m.l.d. per ml. Although the maximum concentration obtained in cultures containing 1, 2 and 5 per cent serum was 75,000 m.l.d. per ml., it was found that all the animals infected with the various dilutions of the culture containing 1 per cent serum succumbed to the infection. One per cent serum concentration gave consistently good results and was generally adopted. A concentration of 2 per cent of sheep serum, however, was found to give better results in cultures with a medium containing 1·5 per cent sheep-brain extract, 2·5 per cent glycine and 0·15 per cent peptone without the addition of fresh young guinea-pig brain tissue and was always used with the above medium.

5. *Effect of incubating cultures under aerobic and strict anaerobic conditions.*

The following experiments were carried out to determine whether cultivation under aerobic and anaerobic conditions had any effect on the virus content of cultures grown in different media :—

Experiment A.—Each of the flasks A and B contained 60 ml. of one per cent sheep-brain extract, 1 ml. of sheep serum, 10 ml. each of 1·5 per cent glycine and peptone, and 9 ml. of distilled water. An emulsion of the brains of two young guinea-pigs and 18 ml. of the supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain were added to each flask. Flask A was incubated aerobically and flask B under strict anaerobic conditions in a McIntosh and Fildes' jar. The virus content of the inoculum diluted 1 in 6 and that of the supernatants in flasks A and B after 12 hours' incubation at 37°C. were titrated in guinea-pigs. The results are given in Table X :—

TABLE X.

	Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.					
		1/2,000	1/2,666	1/10,000	1/15,000	1/20,000	1/30,000
Virus inoculum diluted 1 in 6 with water.	...	S, S	S, S	—	—	—	—
Flask A (aerobic)	12	—	—	6/8, S	8/10, S	—	—
Flask B (anaerobic)	12	—	—	6/8, 7/8	6/8, 7/9	10/12, S	7/10, S

Comment.—The initial virus content of the flasks before incubation was less than 10,000 m.l.d. per ml. After incubation for 12 hours at 37°C. the virus content of the culture in flask A incubated aerobically was 75,000 m.l.d. per ml., whereas the virus content of the culture in flask B incubated under strict anaerobic conditions was 150,000 m.l.d. per ml. This indicated that although virus showed evidence of multiplication under both aerobic and anaerobic conditions, the concentration of the virus in cultures incubated under strict anaerobic conditions was higher.

Experiment B.—The above experiment was repeated with a medium consisting of 120 ml. of one per cent sheep-brain extract, 2 ml. of sheep serum, 25 ml. of 20 per cent glycine, 20 ml. of 1·5 per cent peptone and 13 ml. of distilled water. To each of the flasks A and B containing the above medium were added an emulsion of the brains of four young guinea-pigs and 36 ml. of the supernatant from a 20 per cent suspension of fixed virus sheep brain. Flask A was incubated aerobically and flask B anaerobically. The virus content of the

supernatants from flasks A, B and C after incubation at 37°C. for 12 hours were titrated in guinea-pigs. The results are given in Table VIII:—

TABLE VIII.

	Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.					
		1/500	1/1,000	1/2,500	1/5,000	1/7,500	1/10,000
Virus inoculum diluted 1 in 6 with water.	...	9/10, 9/10	9/10, 9/10	S, S	—	—	—
Flask A (without serum).	12	—	—	7/8, 7/9	8/10, S	S, S	8/9, 9/10
Flask B (with 5% serum).	12	—	—	6/8, 6/8	6/8, 6/8	8/10, 8/11	7/9, S
Flask C (with 10% serum).	12	—	—	—	6/8; 6/8	7/8, 7/9	S, S

Comment.—The virus content of the inoculum diluted 1 in 6 representing the initial concentration of the virus in the flasks was 5,000 m.l.d. per ml. The virus content of the cultures in flasks A and B containing no serum and 5 per cent serum respectively after incubation for 12 hours at 37°C. was 50,000 m.l.d. per ml. The virus content of the culture in flask C containing 10 per cent serum was only 37,500 m.l.d. per ml. This indicated that a concentration of 10 per cent of sheep serum in the medium was not necessary. Although good results were obtained with medium containing no serum and 5 per cent serum, it was found that, while all the dilutions of the culture containing 5 per cent serum proved to be regularly infective, the infectivity of the various dilutions of the culture containing no serum was irregular. Therefore, experiments were undertaken to find out whether a concentration of serum between 0 and 5 per cent would give consistent results.

Experiment B.—The above experiment was repeated with media containing 1, 2 and 5 per cent sheep serum. Flask A contained 120 ml. of sheep-brain extract, 20 ml. each of 1.5 per cent glycine and peptone, 18 ml. of distilled water and 2 ml. of sheep serum. Flask B contained the same quantities of brain extract, glycine and peptone with 16 ml. of distilled water and 4 ml. of sheep serum. Other constituents being the same flask C contained 10 ml. of distilled water and 10 ml. of sheep serum. An emulsion of the brains of four young guinea-pigs were added to each flask just before inoculation of the virus. Thirty-six ml. of the supernatant from a centrifugalized suspension of 20 per cent fixed virus sheep brain were added to each flask. The initial concentration of the virus in the flasks represented by the virus content of the inoculum diluted 1 in 6 was determined. After incubation for 12 hours at 37°C., the contents of each flask were centrifuged separately for 10 minutes and the virus content of the supernatants titrated in guinea-pigs. The results are given in Table IX:—

TABLE IX.

	Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.		
		1/1,000	1/10,000	1/15,000
Virus inoculum diluted 1 in 6 with water.	...	S, S	—	—
Flask A (with 1% serum)	12	—	7/8, 7/8	8/10, 8/10
Flask B (with 2% serum)	12	—	7/8, 7/8	9/11, S
Flask C (with 5% serum)	12	—	6/8, 6/8	8/9, S

Comment.—It will be seen from Table IX that the initial virus content of the flasks was less than 5,000 m.l.d. per ml. Although the maximum concentration obtained in cultures containing 1, 2 and 5 per cent serum was 75,000 m.l.d. per ml., it was found that all the animals infected with the various dilutions of the culture containing 1 per cent serum succumbed to the infection. One per cent serum concentration gave consistently good results and was generally adopted. A concentration of 2 per cent of sheep serum, however, was found to give better results in cultures with a medium containing 1·5 per cent sheep-brain extract, 2·5 per cent glycine and 0·15 per cent peptone without the addition of fresh young guinea-pig brain tissue and was always used with the above medium.

5. *Effect of incubating cultures under aerobic and strict anaerobic conditions.*

The following experiments were carried out to determine whether cultivation under aerobic and anaerobic conditions had any effect on the virus content of cultures grown in different media :—

Experiment A.—Each of the flasks A and B contained 60 ml. of one per cent sheep-brain extract, 1 ml. of sheep serum, 10 ml. each of 1·5 per cent glycine and peptone, and 9 ml. of distilled water. An emulsion of the brains of two young guinea-pigs and 18 ml. of the supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain were added to each flask. Flask A was incubated aerobically and flask B under strict anaerobic conditions in a McIntosh and Fildes' jar. The virus content of the inoculum diluted 1 in 6 and that of the supernatants in flasks A and B after 12 hours' incubation at 37°C. were titrated in guinea-pigs. The results are given in Table X :—

TABLE X.

		FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.					
	Number of hours at 37°C.	1/2,000	1/2,666	1/10,000	1/15,000	1/20,000	1/30,000
Virus inoculum diluted 1 in 6 with water.	...	S, S	S, S	—	—	—	—
Flask A (aerobic)	12	—	—	6/8, S	8/10, S	—	—
Flask B (anaerobic)	12	—	—	6/8, 7/8	6/8, 7/9	10/12, S	7/10, S

Comment.—The initial virus content of the flasks before incubation was less than 10,000 m.l.d. per ml. After incubation for 12 hours at 37°C. the virus content of the culture in flask A incubated aerobically was 75,000 m.l.d. per ml., whereas the virus content of the culture in flask B incubated under strict anaerobic conditions was 150,000 m.l.d. per ml. This indicated that although virus showed evidence of multiplication under both aerobic and anaerobic conditions, the concentration of the virus in cultures incubated under strict anaerobic conditions was higher.

Experiment B.—The above experiment was repeated with a medium consisting of 120 ml. of one per cent sheep-brain extract, 2 ml. of sheep serum, 25 ml. of 20 per cent glycine, 20 ml. of 1·5 per cent peptone and 13 ml. of distilled water. To each of the flasks A and B containing the above medium were added an emulsion of the brains of four young guinea-pigs and 36 ml. of the supernatant from a 20 per cent suspension of fixed virus sheep brain. Flask A was incubated aerobically and flask B anaerobically. The virus content of the

inoculum diluted 1 in 6 and that of the supernatants of the twelfth-hour cultures in flasks A and B were titrated in guinea-pigs. The results are given in Table XI:—

TABLE XI.

		FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.					
	Number of hours at 37°C.	1/2,666	1/3,333	1/60,000	1/80,000	1/100,000	1/150,000
Virus inoculum diluted 1 in 6 with water.	...	6/7, 10/12	S, S	—	—	—	—
Flask A (aerobic)	12	—	—	6/9, 7/10	8/11, S	S, S	S, S
Flask B (anaerobic)	12	—	—	—	7/9, 7/9	3/4, 7/10	8/10, S

Comment.—It will be seen from Table XI that the initial virus content of the flasks before incubation was 13,330 m.l.d. After incubation for 12 hours at 37°C. the virus content of the culture in flask A incubated aerobically was 400,000 m.l.d. per ml., whereas the virus content of the culture in flask B incubated under strict anaerobic conditions was 750,000 m.l.d. per ml., indicating that cultivation under strict anaerobiosis was more favourable for the multiplication of the virus.

Experiment C.—The experiment was repeated with a medium consisting of 7.5 ml. of 10 per cent steamed sheep-brain extract, 1 ml. of sheep serum, 6.25 ml. of 20 per cent glycine, 5 ml. of 1.5 per cent peptone and 25.25 ml. of distilled water without the addition of fresh guinea-pig nerve tissue. Thirty-six ml. of the supernatant from a 20 per cent suspension of fixed virus sheep brain were added to each of the flasks containing the above medium. Flask A was incubated aerobically and flask B anaerobically. The virus content of the inoculum diluted 1 in 6 and that of the twenty-fourth-hour cultures in flasks A and B were titrated in guinea-pigs. The results are given in Table XII:—

TABLE XII.

		FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.				
	Number of hours at 37°C.	1/2,666	1/3,333	1/100,000	1/200,000	1/300,000
Virus inoculum diluted 1 in 6 with water.	...	5/7, 8/10	S, S	—	—	—
Flask A (aerobic)	24	—	—	10/12, S	9/11, S	S, S
Flask B (anaerobic)	24	—	—	6/8, 7/9	6/8, 7/9	6/8, 7/10

Comment.—It will be seen from Table XII that the initial virus content of the flasks was 13,330 m.l.d. per ml. After incubation for 24 hours at 37°C. the virus content of the flask A incubated aerobically was 1,000,000 m.l.d. per ml., while that of flask B incubated anaerobically was 1,500,000 m.l.d. per ml., indicating that strict anaerobiosis is more favourable for the multiplication of the virus.

6. Attempts to cultivate rabies virus in a cell-free medium.

In the early stages of the work an emulsion of the brain of one young guinea-pig in 5 ml. of distilled water was added to every 45 ml. of a medium containing 30 ml. of one per cent sheep-brain extract, 0.5 ml. of sheep serum, .5 ml. each of 1.5 per cent glycine and peptone, and 4.5 ml. of distilled water. The concentration of the virus in the twelfth-hour culture was of the order of 75,000 m.l.d. per ml. Later studies showed that by increasing the glycine content of the medium to 2.5 per cent much higher concentrations of the virus could be obtained in 12 hours.

Attempts were now made to determine if it was possible to eliminate from the medium fresh nerve tissue without affecting the virus titre of the culture. In order to avoid depletion of constituents probably essential for the growth of the virus and provided by guinea-pig brain tissue, it was considered desirable to increase the concentration of steamed cell-free sheep-brain extract to compensate for the deficiency.

Experiment A.—To each of the flasks A, B, C, D and E were added 0.5 ml. of sheep serum, 6.25 ml. of 20 per cent glycine and 5 ml. of 1.5 per cent peptone. To flask A was added 3 ml. of 10 per cent steamed brain extract, 30.25 ml. of distilled water and an emulsion of the brain of one young guinea-pig. Flask B contained the same constituents without the guinea-pig nerve tissue. To flask C was added 7.5 ml. of 10 per cent sheep-brain extract and 25.75 ml. of distilled water without fresh nerve tissue. To flasks D and E were added 15 ml. of 10 per cent sheep-brain extract and 18.25 ml. of distilled water. Fresh nerve tissue was not added to flask D, while 10 drops of an emulsion of the brain of a young guinea-pig in 5 ml. of distilled water were added to flask E. Thus, the final concentration of sheep-brain extract in the media in flasks A, B, C, D and E were 0.6, 0.6, 1.5, 3 and 3 per cent respectively. An emulsion of the brain of a young guinea-pig was added to flask A, while only a few drops of brain emulsion were added to flask E, the latter to find out whether the addition of a small amount of fresh nerve tissue was absolutely essential to start the multiplication of the virus in the culture medium. Nine ml. of the supernatant from a 20 per cent fixed virus sheep brain were added to each flask and the flasks incubated at 37°C. for 12 hours. The virus content of the inoculum diluted 1 in 6 and the supernatants from the flasks at the end of 12 hours' incubation were titrated in guinea-pigs. The results are given in Table XIII:—

TABLE XIII.

	Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0.2 ML. OF CULTURE IN DILUTIONS.						
		1/2,000	1/2,666	1/3,333	1/15,000	1/30,000	1/45,000	1/60,000
Virus inoculum diluted 1 in 6 with water.	...	7/9, 9/12	11/12, S	S, S	—	—	—	—
Flask A (0.6% sheep- brain extract with guinea-pig brain).	12	—	—	—	—	—	6/9, 7/10	8/10, S
Flask B (0.6% sheep- brain extract with- out guinea-pig brain).	12	—	—	—	S, S	S, S	S, S	—
Flask C (1.5% sheep- brain extract with- out guinea-pig brain).	12	—	—	—	7/9, 7/9	6/9, 9/11	6/9, 7/10	—
Flask D (3% sheep- brain extract without guinea-pig brain).	12	—	—	—	7/9, 7/9	7/9, 8/9	7/9, S	—
Flask E (3% sheep- brain extract with ten drops of an emul- sion of guinea-pig brain).	12	—	—	—	9/12, S	9/11, 9/11	8/10, S	—

Comment.—It will be seen from Table XIII that the initial virus content of the flasks was 13,330 m.l.d. per ml. At the end of 12 hours' incubation the virus content of flask A containing 0·6 per cent sheep-brain extract and young guinea-pig nerve tissue was 300,000 m.l.d. per ml., while that of flask B containing the same percentage of sheep-brain extract without the addition of nerve tissue was less than 75,000 m.l.d. as it was not infective in a dilution of 1 in 15,000, the lowest dilution tested. The virus content of the cultures in flasks C and D containing 1·5 and 3 per cent sheep-brain extract without guinea-pig nerve tissue and that of flask E containing 3 per cent sheep-brain extract and a few drops of young guinea-pig nerve tissue was over 225,000 m.l.d. per ml. Unfortunately, the infectivity of cultures in flasks C, D and E in dilutions higher than 1 in 45,000 were not tried and, therefore, the highest concentration of the virus reached in the different flasks could not be determined. The experiment, however, clearly showed that it was possible to cultivate rabies virus in a medium containing sheep-brain extract, sheep serum, glycine and peptone without the addition of fresh young guinea-pig nerve tissue. The finding that all the animals infected with various dilutions of the culture in flask C containing 1·5 per cent sheep-brain extract succumbed to the infection suggested that a concentration of 1·5 per cent sheep-brain extract in the medium was quite adequate.

Experiment B.—The experiment was repeated in order to determine the highest concentration of the virus that could be obtained with the above medium under aerobic and anaerobic conditions.

To each of the flasks A and B were added 7·5 ml. of 10 per cent steamed sheep-brain extract, 1 ml. of sheep serum, 6·25 ml. of 20 per cent glycine, 5 ml. of 1·5 per cent peptone and 25·25 ml. of distilled water. Nine ml. of the supernatant from a 20 per cent suspension of fixed virus sheep brain were added to each of the flasks. Flask A was incubated aerobically and flask B under strict anaerobiosis. The initial virus content of the inoculum diluted 1 in 6 and the virus content of the culture in flasks A and B after 24 hours' incubation at 37°C. was titrated in guinea-pigs. The results are given in Table XII.

Comment.—It will be seen from Table XII that the initial virus content of the cultures in the flasks was 13,330 m.l.d. per ml. After incubation for 24 hours at 37°C. the virus content of the culture in flask A incubated aerobically was 1,000,000 m.l.d. per ml., while that of flask B was 1,500,000 m.l.d. per ml. This showed that it was possible to obtain high concentrations of the virus with a culture medium containing 1·5 per cent sheep-brain extract, 2 per cent sheep serum, 2·5 per cent glycine and 0·15 per cent peptone without the addition of young guinea-pig nerve tissue and that the concentration of the virus was higher when the culture was incubated under strict anaerobic conditions than under aerobic conditions.

Experiment C.—In experiment B the maximum dilution of the anaerobic culture tested and found infective was 1 in 300,000. The experiment was repeated to see if the results could be confirmed and whether dilutions higher than 1 in 300,000 would prove infective. The results are given in Table XIV:—

TABLE XIV.

Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.				
	1/2,000	1/2,666	1/3,333	1/300,000	1/500,000
0	6/8, 7/9	S, S	S, S	—	—
24 (anaerobic).	—	—	—	6/8, 7/9	6/8, 6/8

Comment.—It will be seen from Table XIV that the initial concentration of the virus in the flask was 10,000 m.l.d. The virus content of the culture after 24 hours' incubation under anærobic conditions was 2,500,000 m.l.d. per ml., which is at least five times the maximum concentration of virus obtained in the brains of infected sheep used in the manufacture of antirabic vaccine.

Experiment D.—The sheep-brain extract used in the above experiments was prepared by thoroughly grinding normal sheep brain with sterile glass-powder until a smooth paste was obtained which, when examined under the microscope, showed that all cellular structure was crushed. The ten per cent emulsion was centrifuged for two hours at 3,000 r.p.m. and the supernatant steamed for half an hour on each of three consecutive days. Therefore, the sheep-brain extract used in the preparation of the medium did not contain any cells.

The sheep serum used in the medium was passed through a Seitz pad and therefore was cell free.

In addition to glycine, peptone and distilled water the only other constituent added to the culture medium was the virus inoculum. The virus inoculum was also prepared by grinding fixed virus sheep-brain tissue with sterile glass-powder until all cellular structure was crushed. The 20 per cent suspension was centrifuged for half an hour at 3,000 r.p.m. and the top layer of the supernatant was used as the inoculum.

Thus, it will be seen that it was possible to cultivate the virus in a cell-free medium. In order to eliminate the possibility that the virus inoculum might have contained a few intact cells which might have been responsible for the multiplication of the virus, the experiment was repeated using as the inoculum the supernatant from a 20 per cent virus suspension rendered cell free by passing through a filter (Ford Sterimat, grade F.C.B.).

Flask A contained 30 ml. of 10 per cent steamed sheep-brain extract, 4 ml. of sheep serum, 25 ml. of 20 per cent glycine, 20 ml. of 1·5 per cent peptone and 101 ml. of distilled water. A 20 per cent suspension of fixed virus sheep brain prepared under sterile conditions was centrifuged and the supernatant passed through Sterimat F.C.B. pad. Thirty-six ml. of the filtrate were inoculated into 180 ml. of the medium. The initial virus content of the culture before incubation was determined. The flask was incubated under strict anærobic conditions at 37°C. for 24 hours. At the end of 24 hours the virus content of the culture was titrated in guinea-pigs. The results are given in Table XV:—

TABLE XV.

Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.							
	1/2,000	1/3,000	1/3,333	1/50,000	1/100,000	1/150,000	1/200,000	1/300,000
0	11/13, S	6/8, S	8, S	—	—	—	—	—
24 (anærobic)	—	—	—	6/8, 6/9	6/8, 8/10	7/9, 9/10	6/9, S	S, S

Comment.—It will be seen from Table XV that the initial concentration of the virus in the flask before incubation was 15,000 m.l.d. per ml. After incubation for 24 hours at 37°C. under strict anærobic conditions the concentration was 1,000,000 m.l.d. per ml., thus proving that the ætiological agent of rabies can be successfully cultivated in a cell-free medium.

7. Effect of incubation of cultures for 24 hours.

The following experiments were carried out to determine whether the concentration of the virus obtained in cultures grown in various media could be raised by increasing the period of incubation from 12 to 24 hours :—

Experiment A.—A flask containing 120 ml. of one per cent sheep-brain extract, 20 ml. each of 1·5 per cent glycine and peptone, 18 ml. of distilled water, 2 ml. of sheep serum and an emulsion of the brains of four young guinea-pigs was inoculated with 36 ml. of the supernatant from a 20 per cent suspension of fixed virus sheep brain. The initial virus content of the flask was determined. The culture was incubated at 37°C. At the end of 12 hours the culture was thoroughly shaken and 10 ml. pipetted out. This was centrifuged slowly to get rid of gross nerve tissue and the virus content of the supernatant titrated in animals. Similarly, the virus content of the twenty-fourth-hour culture was determined. The results are given in Table XVI :—

TABLE XVI.

Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.		
	1/1,000	1/10,000	1/15,000
0	S, S	—	—
12	—	6/8, 6/8	8/9, 8/9
24	—	7/9, 9/10	S, S

Comment.—It will be seen from Table XVI that, while the virus content of the culture grown in the above medium was 75,000 m.l.d. that of 24 hours' culture was 50,000 m.l.d. per ml. indicating a fall in the titre when the culture was incubated for 24 hours.

Experiment B.—The above experiment was repeated with a medium containing 1·5 per cent sheep-brain extract, 2·5 per cent glycine, 0·15 per cent peptone and distilled water *without the addition of serum and fresh guinea-pig nerve tissue*. The culture was incubated under anaerobic conditions.

Flask A contained 37·5 ml. of 10 per cent sheep-brain extract, 31·25 ml. of 20 per cent glycine, 25 ml. of 1·5 per cent peptone and 131·25 ml. of distilled water. Forty-five ml. of the supernatant from a 20 per cent suspension of fixed virus sheep brain were added to the flask and incubated at 37°C. under strict anaerobiosis. The initial virus content of the flask and that after 12 and 24 hours' incubation were titrated in guinea-pigs. The results are given in Table XVII :—

TABLE XVII.

Number of hours at 37°C.	FATE OF GUINEA-PIGS INOCULATED SUBDURALLY WITH 0·2 ML. OF CULTURE IN DILUTIONS.				
	1/2,000	1/2,666	1/100,000	1/150,000	1/200,000
0	7/9, 7/10	S, S.	—	—	—
12	—	—	8/10, S	S, S	S, S
24	—	—	6/8, 7/10	7/9, 7/9	5/8, S

Comment.—It will be seen from Table XVII that the initial virus content of the flask was 10,000 m.l.d. per ml. The virus content after 12 and 24 hours' incubation was 500,000 and 1,000,000 m.l.d. per ml., respectively, indicating that with the above medium the concentration of the virus obtained after 24 hours' incubation was greater than that obtained after 12 hours' incubation.

8. *pH of the media.*

In the earlier part of the work the medium generally used was that containing 0·6 per cent sheep-brain extract, 1 per cent sheep serum, 0·15 per cent each of glycine and peptone and young guinea-pig brain tissue. The pH of the culture at the start of the experiment after the addition of one part of the supernatant from a 20 per cent suspension of fixed virus sheep brain to 5 parts of the medium was between 7·0 and 7·2. There was no marked change in the pH after incubation for 12 to 24 hours at 37°C. When, however, a medium containing 1·5 per cent sheep-brain extract, 2 per cent sheep serum, 2·5 per cent glycine and 0·15 per cent peptone was used, the pH of the culture before and after incubation was 6·6. In this connection it is interesting to note that the pH of 10 per cent steamed sheep-brain extract used in the medium is 6·5. The question of buffering the medium or adjusting the pH to 7 was therefore not considered necessary.

9. *Effect of serial passage.*

Several attempts were made to passage the virus in a culture medium containing 0·6 per cent sheep-brain extract, 1 per cent sheep serum, 0·15 per cent each of glycine and peptone and fresh young guinea-pig nerve tissue. Transfer to the second passage flask was made by centrifuging the twelfth-hour culture slowly, and then transferring 9 ml. of the supernatant to 45 ml. of the medium in the second flask. This method of serial inoculation of culture media was repeated regularly. The main difficulty encountered in carrying out serial passage of the cultures *in vitro* with the above medium was to keep the cultures sterile. The chief source of contamination appeared to be from the fresh guinea-pig nerve tissue which was added to the medium. The experiments showed that common contaminants such as Gram-positive cocci and bacilli had a deleterious action on the multiplication of the virus.

In one experiment it was possible to carry the virus through three serial passages. The initial virus content of the first passage flask before incubation was less than 5,000 m.l.d. and after 12 hours' incubation 75,000 m.l.d. per ml. The virus content of the second passage flask after 12 hours' incubation was also 75,000 m.l.d., while that of the third passage flask was only 50,000. The virus content of the fourth passage flask was not titrated as it was found to be contaminated. This experiment showed that but for the technical difficulty of keeping the cultures free from outside contaminants it was possible to carry the virus from flask to flask *in vitro* with evidence of multiplication.

The above experiment was repeated with a medium containing 1·5 per cent sheep-brain extract, 2 per cent sheep serum, 2·5 per cent glycine and 0·15 per cent peptone without the addition of fresh nerve tissue. The cultures were incubated at 37°C. under strict anaerobic conditions and inoculation of culture media done at intervals of 24 hours. In this experiment the initial concentration of the virus in the first passage flask was 13,330 m.l.d. and the concentration after 24 hours' incubation anaerobically was 1,500,000 m.l.d. per ml. Serial passage was continued and only the fourth passage culture after 24 hours' incubation was inoculated into guinea-pigs. The culture, although sterile, did not prove to be infective even in 1 in 1,000 dilution indicating that the virus concentration was less than 5,000 m.l.d. The factor or factors responsible for the fall in the titre of the cultures on serial passage are under investigation.

10. *The highest concentration of virus obtained in cultures.*

The highest concentration of virus so far obtained in cultures has been with a medium containing 1·5 per cent steamed sheep-brain extract, 2 per cent sheep serum, 2·5 per cent glycine and 0·15 per cent peptone to which has been added the supernatant from a 20 per

cent suspension of fixed virus sheep brain in the proportion of 1 part to 5 parts of the above medium. The initial virus content of the culture generally varies from 10,000 to 16,665 m.l.d. per ml. But, after incubation for 24 hours at 37°C. the virus content of the culture rises to 2,500,000 m.l.d. per ml.

11. *Immunizing value of culture virus.*

Proof that the culture material was rabies virus was obtained by immunizing guinea-pigs and rabbits intraperitoneally with the culture virus and testing them three weeks later for resistance to subdural injection of fixed virus or intramuscular injection of a virulent strain of street virus. The results of the immunity experiments indicate that when the virus content of the culture vaccine is adjusted to be equal to that of 5 per cent phenol vaccine it affords protection against 1,000 subdural lethal doses and is superior to that obtained with phenol vaccine. The details of the work are presented in another paper (Veeraraghavan, 1946, p. 225 this issue).

DISCUSSION.

It will be clear from the observations recorded that a high concentration of rabies virus can be obtained in cultures *in vitro* in a medium containing 1.5 per cent sheep-brain extract, 2 per cent sheep serum, 2.5 per cent glycine and 0.15 per cent peptone without the addition of fresh young guinea-pig brain tissue. The initial concentration of the virus in cultures with the above medium varies from 10,000 to 16,665 m.l.d. per ml. After 24 hours' incubation at 37°C. under strict anaerobic conditions the titre of the culture rises to at least 2.5 million m.l.d. per ml. indicating that the virus has multiplied in the medium.

There is a marked fall in the concentration of virus when one part of the supernatant from a 20 per cent suspension of fixed virus is added to five parts of distilled water, pH 6.6 and the mixture incubated for 24 hours at 37°C. This observation together with the finding that a high concentration of the virus can be obtained in cultures grown in a cell-free medium clearly indicates that the rise in virus concentration in cultures is not due to a simple process of autolysis, but is the result of actual multiplication of the virus in the culture medium used.

The effect of varying the concentrations of different ingredients used in the culture medium has been studied. In the absence of fresh nerve tissue it was found that a concentration of 1.5 per cent of steamed sheep-brain extract was necessary for the multiplication of the virus. The optimum concentration of glycine necessary was found to be 2.5 per cent, while a concentration of 0.15 per cent peptone considerably helped the multiplication of the virus. Evidence of multiplication of the virus could be obtained in cultures grown in media which did not contain serum. When serum was added to the culture media in concentrations of 1 or 2 per cent the results obtained were, however, much more satisfactory.

The concentration of the virus obtained in cultures incubated for 24 hours at 37°C. under strict anaerobic conditions was much higher than that incubated under aerobic conditions. This observation would explain the fact that deep bites are more dangerous than superficial ones (McKendrick, 1938).

A number of workers have shown that rabies virus can be cultivated in tissue culture. The media used by them invariably contained rabbit-, mouse-, rat- or chick-embryo brain tissue with serum-Tyrode solution. In this paper experimental evidence is presented for the first time to show that the addition of fresh embryonic tissue to the medium can be dispensed with and that it is possible to cultivate the virus in a medium containing no living or growing cells. The fact that the aetiological agent of rabies could be cultivated in a cell-free medium conclusively proves that it is not a virus in the fully accepted sense of the word. This finding lends support to the view put forward by Veeraraghavan (1944, 1945) that an agent other than a virus may be connected with the aetiology of rabies.

The medium is easy to prepare and the technique of cultivation is not difficult. The virus can be cultivated in any quantity provided the proportions of the various ingredients used in the medium are maintained.

The chief difficulty experienced so far in using culture virus as a source of antirabic vaccine has been its low virus content. The concentration of the virus obtained in cultures with 1·5 per cent sheep-brain extract, 2 per cent sheep serum, 2·5 per cent glycine and 0·15 per cent peptone assessed in terms of minimum lethal doses for the guinea-pig has been found to be much higher than that reported by previous workers using mice as experimental animals.

Immunity experiments (Veeraraghavan, 1946, p. 225 this issue) with culture vaccine show that when its virus content is adjusted to be equal to that of 5 per cent phenol vaccine it affords a considerable degree of protection against fixed virus given subdurally or street virus given into the neck muscles. Protection results with the culture vaccine are in no way inferior to that obtained with phenol vaccine.

The virus content of the culture is at least five times the maximum concentration of virus obtained in the brains of infected sheep used in the manufacture of antirabic vaccine and 100 times that present in 5 per cent phenol vaccine. If immunity following vaccination is dependent on the virus content of the vaccine used it should be possible to administer in 1·4 ml. of culture vaccine the virus content of 140 ml. of 5 per cent phenol vaccine at present used in the treatment of class III patients thus reducing considerably (a) the dosage of vaccine administered, (b) the duration of treatment, and (c) the amount of nerve-tissue protein given.

SUMMARY.

1. A simple medium containing steamed sheep-brain extract, sheep serum, glycine and peptone is described for the cultivation of rabies fixed virus *in vitro*. It is possible to obtain in cultures with the above medium at least five times the maximum concentration of virus obtained in the brains of infected sheep used in the manufacture of antirabic vaccine.

2. Cultivation under strict anaerobic conditions is more favourable for the multiplication of the virus than under aerobic conditions.

3. The successful cultivation of the aetiological agent of rabies in a cell-free medium proves that it is not a virus in the accepted sense of the term.

4. Protection afforded by culture vaccine whose virus content is adjusted to be equal to that of 5 per cent phenol vaccine compares favourably with that obtained with phenol vaccine.

5. With the high concentration of the virus obtained in cultures it should be possible to administer in 1·4 ml. the virus content of 140 ml. of 5 per cent phenol vaccine at present used in the treatment of class III patients.

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Addendum.—The following observations were made after the paper was sent for publication :—

The addition of certain accessory factors had a beneficial effect on the growth and multiplication of the virus *in vitro*. Titres as high as 500 million m.l.d. per ml. (as compared with 2·5 million m.l.d. per ml. reported in the paper) were obtained when thiamine hydrochloride, pyridoxine hydrochloride, calcium pantothenate, nicotinic acid and riboflavine were added in a concentration of 2 µg. per ml. to the medium containing 1·5 per cent steamed sheep-brain extract, 2 per cent sheep serum, 2·5 per cent glycine, 0·002 per cent tryptophane and 0·15 per cent peptone.

With the above medium containing all the accessory factors and Tyrode solution, used as a diluent instead of distilled water, but, *without the addition of normal sheep-brain extract*, it was possible to step up the titre to 100 million m.l.d. per ml.

The use of Waring blender for emulsification of brain was found to be of definite advantage. When a blender was used for the preparation of sheep-brain extract and virus inoculum it was possible to obtain a titre of 25 million m.l.d. per ml. with a medium containing 1·5 per cent steamed sheep-brain extract, 2 per cent sheep serum, 2·5 per cent glycine and 0·15 per cent peptone. The cultures were incubated for 48 hours under strict anaerobic conditions.

The details of the work will be published later.

N. VEERARAGHAVAN,
 16th October, 1946.

STUDIES ON ANTIRABIC IMMUNIZATION WITH CULTURE VACCINE.

BY

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INTRODUCTION.

SINCE rabies vaccine was first used it has been subjected to many types of experimental tests to determine its immunizing properties. Webster (1939a) in a review of the subject emphasized the general lack of positive results in the light of statistical analysis of published protocols. Webster (1939b) evolved a quantitative test for measuring the immunizing potency of antirabic vaccine. Employing this technique he found that commercial vaccines inactivated with phenol and prepared for the treatment of man, in general, failed to protect mice.

The chief defects with 5 per cent Semple's vaccine used in India are the following :—

1. The virus content of the vaccine is not known. Quantitative estimations of the virus content of the brains of sheep used in the manufacture of antirabic vaccine show a considerable degree of variation. Habel (1940a, 1940b, 1941) has studied the various factors influencing the virus content of the brains of animals used in the preparation of antirabic vaccine and attention to the details enumerated by him should reduce variations in the titre to reasonable limits.

2. The antirabic vaccine contains a large amount of nerve tissue which is unlikely of itself to have any antigenic value. When vaccines containing killed virus are used in treatment, the normal nerve tissue present in them is believed to be the cause of neuromyolytic accidents (Remlinger, 1937).

3. The vaccine is administered in large doses over relatively long periods (up to 10 ml. daily for 14 days) and it is, therefore, cumbersome both from the point of view of the patient and medical attendant.

One of the important problems in rabies is to evolve a refined vaccine of greater immunizing value, which could be given in smaller doses over a shorter period of time and which would be free from possible by-effects such as paralytic accidents. It is evident that most of the defects in Semple's vaccine could be remedied if the virus could be obtained in high concentrations in cultures *in vitro*. Webster and Clow (1936, 1937) reported the successful cultivation of the rabies virus *in vitro* and the successful immunization of animals with culture virus. Kligler and Bernkopf (1938) found that formolized tissue-culture virus possessed considerable immunizing properties. Hodes, Webster and Lavin (1940) reported that rabies culture virus exposed to the rays of a mercury vapour lamp lost its virulence and yet retained considerable immunizing potency. But Webster and Casals (1941), after a considerable amount of work on the subject, came to the conclusion that from a practical point of view culture virus was not a satisfactory source of rabies vaccine due chiefly to its low content of virus.

Veeraraghavan (1946, p. 207 this issue) reported that it was possible to obtain high concentrations of the virus in cultures with a medium containing sheep-brain extract, sheep serum, glycine and peptone with or without the addition of young guinea-pig nerve tissue. With the above technique it has been possible to prepare a vaccine which is free from gross nerve tissue and contains only a small fraction of the nerve-tissue protein present in phenol vaccine. The virus content of the vaccine is high and can be easily standardized. The culture vaccine administered in small doses has considerable immunizing properties. This paper records the experimental data connected with the preparation and standardization of culture vaccine together with the results of immunity experiments.

trichloroacetic acid for the precipitation of the protein fraction. The results are given in Table II:—

TABLE II.

				Total nitrogen in mg. per 100 ml.	Non-protein nitro- gen in mg. per 100 ml.	Protein nitrogen in mg. per 100 ml. (by difference).
Medium :						
Sheep-brain extract	10.0	2.0	8.0
Sheep serum	10.0	1.0	9.0
Glycine	32.0	32.0	...
Peptone	26.0	26.0	...
Fixed virus sheep brain (20%) used as inoculum	...			32.0	15.0	17.0
Added guinea-pig brain tissue	20.0*	4.0*	16.0
Total in culture vaccine	130.0	80.0	50.0
5% phenol vaccine	63.0	14.0	49.0

* Calculated figures.

Comment.—It will be seen from Table II that the protein-nitrogen content of the culture vaccine and 5 per cent Semple's vaccine are almost equal. But 20 per cent of the protein nitrogen of the culture vaccine is due to the sheep serum used in the medium. Thus, although the culture vaccine contains at least three times the concentration of virus present in Semple's vaccine, its protein-nitrogen content, due chiefly to the nerve tissue present in it, is only 80 per cent of the latter.

The non-protein nitrogen content of the culture vaccine is greater than that of Semple's vaccine and this is due mainly to glycine and peptone.

5. Immunity experiments.

The immunizing properties of culture vaccine prepared according to the method described above were investigated in parallel with 5 per cent phenol vaccine. Guinea-pigs and rabbits were used and the technique employed for assessing the immunizing potency of the vaccines was a modification of the mouse test described by Habel (1940b) adapted for the guinea-pig.

(a) Experiments with fixed virus subdurally as the test dose.

Experiment I.—Culture vaccine was prepared according to the method described above. The virus content of the twelfth-hour culture proved to be 75,000 m.l.d. per ml. Incubation of the culture for 72 hours at 37°C. after the addition of 0.5 per cent carbolic acid had rendered it non-infective to guinea-pigs when given subdurally. This vaccine was used in the treatment of animals along with a brew of 5 per cent Semple's vaccine prepared for the treatment of human patients.

Fifty-seven guinea-pigs, each weighing between 350 g. and 400 g., were divided into five groups, four of 12 each and one of 9. The first group (12 animals) was treated with culture vaccine diluted one part in seven parts of normal saline. The second group (12 animals) was treated with culture vaccine diluted one part in three and a half parts of normal saline. The

third group (12 animals) received undiluted culture vaccine and the fourth group (12 animals) received 5 per cent phenol vaccine. The treatment consisted of six 1-ml. doses of the vaccine given intraperitoneally on alternate days. The last group (9 animals) was not treated and served as control.

On the 21st day after the commencement of treatment all the animals were given subdurally 0.2 ml. of serial tenfold dilutions in distilled water of supernatant from a centrifugalized 10 per cent suspension of fixed virus passaged in rabbits. The treated groups of animals were divided into batches of four and the untreated group into batches of three. All the animals in each batch received a particular dilution of the virus. The protection conferred by various methods of treatment was estimated in terms of subdural lethal doses. The results are summarized in Table III:—

TABLE III.

Group.	Dose of vaccine.	FATE OF GUINEA-PIGS INOCULATED ON THE 21ST DAY AFTER COMMENCING TREATMENT WITH 0.2 ML. OF FIXED VIRUS SUBDURALLY IN DILUTIONS.				Number of m.l.d. + protection (dilution = 1 m.l.d.).
		1/100	1/1,000	1/10,000	1/100,000	
I	Six doses of 1 ml. of 1 in 7 dilution of culture vaccine, intraperitoneally on alternate days.	3/4*	2/4	1/4	—	55
II	Six doses of 1 ml. of 1 in 3.5 dilution of culture vaccine intraperitoneally on alternate days.	2/4	0/4	0/4	—	550
III	Six doses of 1 ml. of undiluted culture vaccine intraperitoneally on alternate days.	2/4	0/4	0/4	—	550
IV	Six doses of 1 ml. of 5 per cent phenol vaccine intraperitoneally on alternate days.	3/3	3/4	0/4	—	55
V	Untreated controls	...	—	3/3	3/3	1/3 (1/55,000)

Note.—3/4* = 3 out of 4 guinea-pigs injected died of rabies.

+ = All 50 per cent end points estimated by the method of Reed and Muench (1938).

— = Dilution not tested.

Comment.—It will be seen from Table III that culture vaccine containing the same or higher concentration of virus than that present in 5 per cent phenol vaccine affords considerable degree of protection to guinea-pigs under conditions of the test described. The protection afforded by 5 per cent phenol vaccine used in this experiment and culture vaccine diluted 1 in 7, whose virus content was half that of phenol vaccine, was rather low.

Experiment II.—The virus content of the culture vaccine used in the experiment was 75,000 m.l.d. per ml. This vaccine was used in the treatment of animals along with a brew of 5 per cent phenol vaccine prepared for the treatment of human patients.

Thirty-four guinea-pigs, each weighing about 700 g., were divided into four groups, three of 10 each and one of 4. The first group (10 animals) was treated with culture vaccine diluted one part in 7 parts of saline. One of the animals in this group died of sepsis on the fourth day after the commencement of treatment. The second group (10 animals) was treated with culture vaccine diluted 1 in 3.5 with saline. One animal in this group also died of sepsis on

the ninth day after the commencement of treatment. The third group (10 animals) received 5 per cent phenol vaccine. The treatment consisted of six 1.75-ml. doses of the vaccine given intraperitoneally on alternate days. The last group (4 animals) was not treated and served as control.

On the 21st day after the commencement of treatment all the animals were given subdurally 0.2 ml. of a 1 in 1,000 dilution in distilled water of rabbit fixed virus. The virus inoculum was prepared by diluting one part of the supernatant from a centrifugalized 10 per cent suspension of fixed virus in 99 parts of distilled water. The results are given in Table IV :—

TABLE IV.

Group.	Method of treatment.	Number treated.	Number died of rabies.	Average incubation period in days.	Mortality per cent.
I	Six doses of 1.75 ml. of 1 in 7 dilution of culture vaccine intraperitoneally on alternate days.	9	3	6	33
II	Six doses of 1.75 ml. of 1 in 3.5 dilution of culture vaccine intraperitoneally on alternate days.	9	2	9.5	22
III	Six doses of 1.75 ml. of 5% phenol vaccine intraperitoneally on alternate days.	10	4	17	40
IV	Untreated controls 	4	4	6.5	100

Comment.—It will be seen from Table IV that 3 out of 9 animals treated with culture vaccine diluted 1 in 7, 2 out of 9 animals treated with culture vaccine diluted 1 in 3.5, 4 out of 10 animals treated with phenol vaccine and all the controls died. The results indicate that the protection afforded by culture vaccine diluted 1 in 3.5, whose virus content is equal to that of phenol vaccine, is in no way inferior to that obtained with phenol vaccine.

Experiment III.—The brain of a sheep completely paralysed after subdural inoculation with rabies fixed virus was removed aseptically. The virus content of the brain was 400,000 m.l.d. Culture vaccine was prepared from one half of the brain and 5 per cent phenol vaccine from the other half. The virus content of the culture was 75,000 m.l.d. per ml. It was diluted 1 in 3.5 with normal saline so that the virus content of the culture vaccine was about the same as that of 5 per cent phenol vaccine prepared from the same brain. A group of animals was also treated with a pooled vaccine prepared by mixing equal quantities of phenol vaccine prepared from the brains of 20 different sheep.

Eighty-six animals each weighing about 400 g. were divided into four groups, three of 22 each and one of 20. The first group (22 animals) was treated with culture vaccine diluted 1 in 3.5. The second group (22 animals) was treated with 5 per cent phenol vaccine prepared from the same infected sheep brain used for the preparation of culture vaccine. The third group (22 animals) was treated with pooled phenol vaccine. The treatment consisted of six 1-ml. doses of the vaccine given intraperitoneally on alternate days. The last group was not treated and served as control.

On the 21st day after the commencement of treatment all the treated animals and 11 out of 20 of the untreated controls were given 0.2 ml. of a 1 in 1,000 dilution of fixed virus passaged in rabbits in distilled water. The virus inoculum was prepared by diluting one part of the supernatant from a centrifugalized 10 per cent suspension of fixed virus in 99 parts of distilled water.

The remaining 9 animals were divided into three equal groups. The animals in the first group were given 0.2 ml. subdurally of a 10^{-4} dilution of fixed virus. The second group received 0.2 ml. of 10^{-5} dilution and the third group 0.2 ml. of a 10^{-6} dilution subdurally. The results are summarized in Table V:—

TABLE V.

Group.	Dose of vaccine.	FATE OF GUINEA-PIGS INOCULATED ON THE 21ST DAY AFTER COMMENCING TREATMENT WITH 0.2 ML. OF FIXED VIRUS SUBDURALLY IN DILUTIONS.				Number of m.l.d. protection.
		10^{-3}	10^{-4}	10^{-5}	10^{-6}	
I	Six doses of 1 ml. of 1 in 3.5 dilution of culture vaccine intraperitoneally on alternate days.	5/22*	—	—	—	1,000
II	Six doses of 1 ml. of 5% phenol vaccine prepared from the same brain intraperitoneally on alternate days.	12/22	—	—	—	<1,000
III	Six doses of 1 ml. of pooled 5% phenol vaccine intraperitoneally on alternate days.	15/22	—	—	—	<1,000
IV	Untreated controls	... 11/11	3/3	3/3	0/3	...

Note.—5/22* = 5 out of 22 guinea-pigs injected died of rabies.
— = Dilution not tested.

Comment.—It will be seen from Table V that 5 out of 22 animals treated with culture vaccine, 12 out of 22 animals treated with phenol vaccine prepared from the same brain used for the preparation of culture vaccine, and 15 out of 22 animals treated with pooled phenol vaccine died of rabies. All the untreated controls inoculated with 10^{-3} , 10^{-4} and 10^{-5} dilutions of the virus succumbed to the infection indicating that the test dose contained at least 1,000 minimum subdural lethal doses. The highest infective dilution of sheep brain used in the preparation of phenol vaccine and culture vaccine was 1 in 80,000 showing that the virus content of the phenol vaccine used in this experiment was the highest that is ordinarily obtained. The results indicate that the protection afforded by culture vaccine whose virus content is adjusted to be equal to that of phenol vaccine with a high virus content is superior to that obtained with phenol vaccine. The immunizing value of pooled phenol vaccine used in this experiment was rather low.

(b) *Experiment with street virus given intramuscularly as the test dose.*

Experiment.—Fifty-one rabbits, each weighing about 1,300 g., were divided into three groups of 17 each. The first group (17 animals) was treated with culture vaccine used in the previous experiment diluted 1 in 3.5. The second group (17 animals) was treated with pooled 5 per cent phenol vaccine obtained by mixing equal quantities of the vaccine prepared from 20 different infected sheep brains. The third group (17 animals) was not treated and served as control. The treatment consisted of six 3-ml. doses of the vaccine given intraperitoneally on alternate days.

A strain of rabies street virus obtained from a dog was given subdurally into a guinea-pig. The animals showed signs of furious rabies on the seventh day and died on the ninth day. Ten

ml. of the supernatant from a centrifugalized 10 per cent suspension of the above brain were diluted with 90 ml. of distilled water. One ml. of the 1 per cent suspension so obtained was inoculated into the neck muscles of each of the rabbits. The results are given in Table VI :—

TABLE VI.*

Group.	Method of treatment.	Number treated.	Number died of rabies.	Average incubation period in days.	Mortality, per cent.
I	Six doses of 3 ml. of 1 in 3·5 dilution of culture vaccine intraperitoneally on alternate days.	17	0	...	0
II	Six doses of 3 ml. of pooled 5% phenol vaccine intraperitoneally on alternate days.	17	1	74	6
III	Untreated controls	17	17	13	100

Comment.—It will be seen from Table VI that none of the animals treated with culture vaccine, 1 out of 19 animals treated with pooled phenol vaccine and all the untreated controls died of rabies. These results indicate that culture vaccine, which is the same as that used in the previous experiment, affords very good protection against street virus. Protection results with pooled 5 per cent phenol vaccine used in this experiment were equally good.

6. An improved culture vaccine.

All the work described so far in this paper was with a culture vaccine prepared with a medium containing 0·6 per cent sheep-brain extract, 1 per cent sheep serum, 0·15 per cent each of glycine and peptone and fresh young guinea-pig nerve tissue. The concentration of the virus obtained in cultures with the above medium was never more than 75,000 m.l.d. per ml. Subsequent work showed that it was possible to obtain concentrations as high as 2,500,000 m.l.d. per ml. in cultures with a medium containing 1·5 per cent sheep-brain extract, 2 per cent sheep serum, 2·5 per cent glycine and 0·15 per cent peptone without the addition of fresh young guinea-pig nerve tissue (Veeraraghavan, *loc. cit.*). The method of preparation of culture vaccine is given below :—

Thirty ml. of 10 per cent sheep-brain extract, 4 ml. of sheep serum, 25 ml. of 20 per cent glycine, 20 ml. of 1·5 per cent peptone and 101 ml. of distilled water were placed in a flask. Thirty-six ml. of the supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain were added to the flask. The flask was incubated at 37°C. under strict anaerobic conditions in a McKintosh and Fildes' jar. After 24 hours' incubation the virus content of the culture was titrated in guinea-pigs. Adequate amounts of 10 per cent carbolic acid were then added to the culture so that the final concentration of the acid was 0·5 per cent. After the addition of carbolic acid the culture was incubated at 37°C. for 72 hours. After 72 hours' incubation the undiluted culture was inoculated subdurally into at least two guinea-pigs in order to find out if the virus was killed.

With the above technique the addition of fresh young guinea-pig nerve tissue to the medium at the start of the experiment and removal of the same after incubation is dispensed with. The maximum concentration of the virus in cultures with the above medium is obtained after 24 hours' incubation instead of 12 hours which makes the technique convenient from a practical point of view. The virus content of the cultures is generally in the neighbourhood of 2,500,000 m.l.d. per ml. which is about five times the maximum concentration obtained in the brains of infected sheep used in the manufacture of 5 per cent phenol vaccine and 100 times that present in phenol vaccine prepared under ideal conditions. Thus it should be

possible to administer in 1·4 ml. the virus content present in 140 ml. of phenol vaccine at present used in the treatment of class III patients.

Incubation of the cultures for 72 hours at 37°C. after the addition of 0·5 per cent carbolic acid kills the virus. The cultures are rendered avirulent when exposed in a petri dish to the rays of a mercury vapour lamp for 10 minutes, the distance from the lamp being 30 cm. and the height of the liquid column 2 mm.

The vaccine prepared by the above method is free from gross nerve tissue and its protein content compares favourably with that of 5 per cent phenol vaccine. The results of the nitrogen estimations with the culture vaccine are given in Table VII :—

TABLE VII.

				Total nitrogen in mg. per 100 ml.	Non-protein nitro- gen in mg. per 100 ml.	Protein nitrogen in mg. per 100 ml. (by difference).
Medium :						
Sheep-brain extract	11·0	3·0	8·0
Sheep serum	18·0	2·0	16·0
Glycine	416·0	416·0	...
Peptone	24·0	24·0	...
Fixed virus sheep brain (20%) used as inoculum	31·0	15·0	16·0
Total in culture vaccine	500·0	460·0	40·0
5% phenol vaccine	64·0	14·0	50·0

DISCUSSION.

The preparation of the culture vaccines described in this paper is not difficult. All the ingredients used in the culture medium can be readily obtained. The maximum concentration of the virus in cultures with a medium containing 1·5 per cent sheep-brain extract, 2 per cent sheep serum, 2·5 per cent glycine and 0·15 per cent peptone without fresh young guinea-pig brain tissue is obtained in 24 hours. The inactivation of the virus with 0·5 per cent carbolic acid takes three days. The vaccine can be used a fortnight later when animal experiments have shown that it contains the requisite amount of killed virus. This period compares favourably with that required for the preparation of 5 per cent phenol vaccine.

The virus content of the inoculum used for inoculating the cultures has to be high if a satisfactory concentration is to be obtained. The supernatant from a centrifugalized 20 per cent suspension of fixed virus sheep brain has been used as the source of virus inoculum and has given consistently good results. If the culture vaccine is to be prepared in large quantities, large amounts of infected brain tissue will be necessary for inoculating the cultures and rabbits may not be quite suited for the purpose. A convenient method would be to keep the Paris strain of rabies fixed virus passaged in rabbits, infect sheep with the rabbit virus, and use the infected sheep brain as the source of virus inoculum for cultures.

Instead of one per cent carbolic acid used for inactivation of virus in Semple's vaccine only half per cent carbolic acid is employed for killing the virus in the culture vaccine. Since a lesser concentration of carbolic acid is added it is advisable that the culture vaccine is always tested for non-infectivity before use.

Estimations of the virus concentration of the brains of infected sheep used in the manufacture of antirabic vaccine show a marked variation in the virus content. Therefore, the virus content of different brews of phenol vaccine prepared from the brains of different sheep must vary considerably. Assuming that the immunizing efficiency of antirabic vaccine is dependent on its virus content the immunity afforded by a vaccine prepared from a sheep-brain infective in a dilution of 1 in 10,000 would be one-tenth that of a vaccine prepared from a sheep-brain infective in a dilution of 1 in 100,000. Both vaccines contain 5 per cent infected sheep-brain tissue. In the absence of any sound method of standardization of the virus content of Semple's 5 per cent vaccine it would appear desirable that in order to obtain a product of fairly uniform virus content the vaccine prepared from 10 or 20 brains should be pooled and issued as a single brew.

The virus content of the culture vaccine is accurately determined and the vaccine can be standardized in terms of m.l.d. per ml. This is a definite improvement over 5 per cent phenol vaccine where the virus content is unknown and variable. Since the Paris strain of rabies fixed virus is always used in the preparation of antirabic vaccine, an exact idea as to the virus content would serve as a reliable index to the immunizing potency of the vaccine. For instance, if the modified culture vaccine containing not less than 2,500,000 m.l.d. per ml. of killed virus is always used in the treatment there is reasonable likelihood of the immunizing potency of the vaccine being uniform. This would obviate the necessity of regularly testing the immunizing value of each batch of vaccine which would be costly and time consuming. It would be enough if the immunizing potency of the vaccine is tested periodically.

Quantitative estimations show that the virus content of the modified culture vaccine described in this paper is five times the maximum concentration of virus obtained in the brains of infected sheep used in the manufacture of 5 per cent phenol vaccine. Therefore, the virus content of the culture vaccine is 100 times as high as that of 5 per cent phenol vaccine prepared under ideal conditions. Further, all the virus in the culture vaccine is in a relatively free state and readily available for exerting its antigenic value.

The culture vaccine, unlike the 5 per cent phenol vaccine, is free from gross nerve tissue. The protein-nitrogen content of undiluted culture in spite of its high virus content is only 80 per cent that of phenol vaccine. The high non-protein-nitrogen content of the culture vaccine is due chiefly to its glycine content and it is possible that the glycine present in the vaccine may have a beneficial effect as it is administered in large doses in muscular dystrophies with good results.

Immunity experiments with culture vaccine indicate that guinea-pigs treated with culture vaccine, whose virus content is half that of phenol vaccine, show little protection under conditions of experiment described. When the virus content of the culture vaccine is adjusted to be equal to that of 5 per cent phenol vaccine the protection afforded is very high. The immunizing value of undiluted culture vaccine containing about 3.5 times the virus content of phenol vaccine did not appear to be superior to that of culture vaccine containing the virus content equal to that of phenol vaccine. This would suggest that after a certain limit the immunological response of an animal cannot be increased by increasing the amount of virus given.

Webster (1939b) found that commercial vaccine inactivated with phenol or chloroform afforded protection to mice if given intraperitoneally, and in quantities approximating five times the dose advocated per gramme of body-weight in man. Webster and Casals (1940) repeating the work with dogs found that chloroform-killed commercial vaccines immunized dogs fairly well if given intraperitoneally in at least double the standard 5-ml. dose. If given subcutaneously in similarly large doses the results, although less striking, were still significant. In the immunity experiments with guinea-pigs and rabbits described in this paper the dosage of vaccine given was proportional to the weight of animals and was based on the dosage recommended by Webster (1939b) for the immunization of mice. The experimental evidence indicates that when five times the comparable dose per gramme of body-weight of phenol vaccine or culture vaccine with the same virus content is given intraperitoneally to guinea-pigs and rabbits a considerable degree of immunity is produced. These findings are in conformity with those reported by Webster (1939b) in mice and by Webster and Casals (1940) in

dogs. If it is assumed that a proportionate increase in the dosage of 5 per cent phenol vaccine in man would produce a greater degree of immunity the total quantity of vaccine required to be given would be 700 ml. which from the practical point of view is not feasible. But with the culture vaccine containing 100 times the virus content of phenol vaccine, it should be possible to administer the whole quantity in 7 ml. The culture vaccine could be given in 1-ml. doses for 7 days or in one or two doses as future experience with the vaccine may indicate. The dosage and duration of treatment would thus be considerably reduced. Besides the total content of nerve-tissue proteins, generally held to be responsible for post-treatment paralysis, would be 1/50th of that present in 140 ml. of phenol vaccine used in the treatment of class III patients.

The culture vaccine prepared in the manner described above is apparently free from toxicity even when administered intraperitoneally to experimental animals. Animals given culture vaccine subcutaneously showed none of the redness and induration at the site of injection frequently observed after the administration of phenol vaccine.

According to the method described for the preparation of culture vaccine it should be possible to prepare 30 ml. of culture vaccine from one gramme of fixed virus sheep brain and, if the dosage is to be reduced as indicated above, it should be possible to prepare thirty times as many courses of culture vaccine from one sheep as that of phenol vaccine. This would mean a marked reduction in the cost of production.

SUMMARY.

A method for the preparation of culture vaccine in a cell-free medium consisting of steamed sheep-brain extract, sheep serum, glycine and peptone is described. This vaccine is superior to Semple's 5 per cent vaccine in respect of the following:—

1. The method of preparation of the culture vaccine is simple.
2. The virus content of the vaccine can be accurately determined. It is 100 times as high as that present in 5 per cent phenol vaccine prepared under ideal conditions.
3. It is free from gross nerve tissue.
4. It is apparently free from toxic effects.
5. When the virus content of the culture vaccine is adjusted to be equal to that of phenol vaccine it affords a considerable degree of protection which is as good as, if not better than, that obtained with phenol vaccine.
6. Assuming that five times the quantity of virus ordinarily used in treatment with phenol vaccine is necessary for producing adequate immunity in man, it should be possible to give with 7 ml. of undiluted culture vaccine five times the virus content present in 140 ml. of phenol vaccine used in the treatment of class III patients.
7. If the above dosage is adopted, the nerve-tissue protein used in the treatment would be reduced to about 1/50th of that used in the treatment with phenol vaccine.

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Examinations made.—Post-mortem examination was made when an animal died or was sacrificed. In each case the liver, spleen, omentum and lymphatic glands were examined for the presence of any pathological changes, and smears from these organs, stained by the Ziehl-Neelsen method, were examined for the presence of acid-fast bacilli.

Results.—Five of the 6 rats inoculated with the treated suspension died within $6\frac{1}{2}$ months after inoculation and the remaining one was sacrificed after this period. All the animals, except one which died only one week after the inoculation, showed heavy generalized leprosy infection with enlargement of liver, spleen and lymphatic glands, the smears from the affected organs showing a large number of acid-fast bacilli.

All the 10 animals of the control group died within $5\frac{1}{2}$ months after inoculation. All of them, except 2 which died within 2 months of inoculation, showed heavy generalized infection, and smears from the affected organs were strongly positive.

Conclusions.—The macroscopic and microscopic findings made in the two groups of animals were similar, there being no apparent difference either in the degree or the extent of the changes. The penicillin treatment of the suspension of rat-leprosy bacillus had no action in inhibiting or retarding the generalized infection. It can, therefore, be said that penicillin in a concentration of 500 Oxford units per c.c., acting on a saline suspension containing rat-leprosy bacilli for 24 hours at a room temperature of about 20°C ., failed to produce any bacteriostatic or bactericidal effects on these bacilli.

THE *in vivo* ACTION.

The infection of rats.—The suspension of rat-leprosy bacillus prepared for the *in vitro* experiment was also used for this experiment, and the method of injection of the rats was the same. Twenty-four white rats (varying in weight from 95 to 105 grammes) were inoculated and put on penicillin treatment immediately after inoculation. The 10 rats, used as the control for the *in vitro* experiment, served as the control for this experiment also.

The treatment of infected rats.—Immediately after inoculation with rat-leprosy material, the rats were put on penicillin treatment. Each rat received 3 injections daily of 100 units. One injection was given intraperitoneally and the other two intramuscularly into each buttock. The injections were given daily, except on Sundays. In the animals which did not die earlier, the treatment was continued for 184 days.

Dilution and assay of penicillin.—The contents of one ampoule of Penicillin Sodium (Squibb) containing 100,000 Oxford units were dissolved in 100 c.c. of pyrogen-free distilled water (pH 7.2). This solution (containing 1,000 units per c.c.) served as the stock solution. It was divided into twelve bottles, which were stored at a temperature of -20°C . The batch of stock solution prepared on one day served for the 12 subsequent days. On each day one bottle was taken out, and final dilutions were made to give a solution containing 200 units per c.c.

The final dilution was assayed each day to check its potency, and it was found that the solution did not deteriorate for at least 12 days. For the assay a method slightly modified from that of Gallardo (1944) was used. Plates of agar, 6 mm. thick, were seeded with a fresh broth culture of *Staph. aureus*. After drying the plates for half an hour, holes were punched out with a sterile 6 mm. cork-borer, these holes were filled with a solution of penicillin containing 10 units per c.c., and the plates were incubated for 24 hours at 37°C . This dilution produced an area of inhibition of about 30 mm. diameter.

Results.—Post-mortem examination was made when an animal died or was sacrificed. All the 24 animals treated with penicillin died within $7\frac{1}{2}$ months after inoculation, and showed heavy generalized infection with enlargement of liver, spleen and lymphatic glands. Smears from the affected organs showed a large number of acid-fast bacilli.

The changes seen in the group of animals treated with penicillin were the same as in the untreated controls, there being no apparent difference in the degree or extent of pathological changes in the two groups.

Conclusions.—In white rats infected with rat-leprosy material, penicillin given intraperitoneally and intramuscularly in doses of 300 units per day for 184 days, failed to modify the course of the resulting generalized infection.

DISCUSSION.

The above experiments would indicate that penicillin, in the dilutions and the doses used, has no bacteriostatic or bactericidal action on the rat-leprosy bacillus. These results are in keeping with the reports of other workers on the insensitiveness of the acid-fast bacilli to penicillin.

Abraham *et al.* (1941) reported that penicillin had no action *in vitro* on *Myco. tuberculosis* even in a concentration of 1 in 1,000 although it produced complete inhibition of *N. gonorrhœæ* in a dilution of 1 in 2,000,000. Robinson (1943) tested the action of penicillin on *Myco. tuberculosis* (avian) both *in vitro* and *in vivo*. His results indicated that penicillin had no action on the avian tubercle bacillus.

Smith and Emmart (1944) confirmed that penicillin did not inhibit the growth of *Myco. tuberculosis*. Linhares (1944) observed that penicillin had no effect on the course of rat leprosy.

The failure of penicillin to influence the course of human leprosy has been reported recently by Faget and Pogge (1945). They report that in lepromatous cases of leprosy, intramuscular injections of penicillin in doses of 50,000 to 320,000 units per day, and continued for 4 to 36 days, had no effect on the disease.

SUMMARY.

Experiments are described which indicate that penicillin has no bactericidal or bacteriostatic action on the rat-leprosy bacillus either *in vitro* or *in vivo*.

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SEROLOGICAL STUDIES IN A CASE OF ACUTE HÆMORRHAGIC JAUNDICE.*

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INTRODUCTION.

THERE is as yet no record of the undoubted occurrence of a case of yellow fever from India. This paper describes a case of acute hæmorrhagic jaundice in a Bengali girl in which the clinical picture and the serological findings were such that, had the child been resident in an area of yellow-fever endemicity, a diagnosis of yellow fever would undoubtedly have been made.

The risk of yellow fever being introduced into India is well known and strict precautions are taken at air and sea ports to prevent such an occurrence. Special 'diagnostic units' are maintained in a state of readiness to investigate the nature of any suspected case. The findings in the case to be described below are, therefore, of considerable interest and importance.

CLINICAL HISTORY OF THE CASE.

The patient, a Bengali girl of eleven years of age, was resident in Calcutta. No history of previous illness was obtained but it was ascertained that she had lived all her life in Bengal.

The girl had enjoyed perfect health until 16th July, 1939, when she suddenly succumbed to a severe illness characterized by fever (102·6°F.), severe headache, and pains in the body and limbs, especially in the leg muscles. On 19th July, she developed abdominal pains with troublesome nausea and, on the following day, persistent vomiting. The vomit at first contained streaks of altered blood and later typical 'coffee grounds'. Hæmorrhages occurred from the nose, under the conjunctiva, and into the skin being most pronounced around the sites of needle punctures where injections had been given. Intense jaundice developed and between 21st and 23rd July the patient became delirious and semi-conscious. Consciousness was regained on 25th July and, after a protracted convalescence, the patient made a complete recovery.

RESULTS OF LABORATORY DIAGNOSTIC TESTS.

The clinical manifestations of the case suggested a severe attack of leptospirosis, a suspicion which was supported by the fact that cases of leptospirosis were known to be occurring in Calcutta at that time.

(1) *Animal inoculation tests for leptospirosis.*—Elaborate animal inoculation tests were carried out in an attempt to establish the leptospiral origin of the disease but all proved to be negative.

(2) *Agglutination tests for evidence of leptospiral infection.*—The patient's serum, obtained on the 9th, 10th, 12th and 20th days of illness, failed to agglutinate all available local strains of *Leptospira icterohæmorrhagiae* as well as other strains of different serological types originally isolated from the Andaman Islands.

* It is contrary to the Editorial policy of the *Indian Journal of Medical Research* to publish case reports of this kind but in view of the special interest and importance attaching to this case an exception to the general rule has been made.—*Editor*.

† Since this paper was received for publication we have to deplore the death of Dr. B. M. Das Gupta.—*Editor*.

A sample of the serum obtained on 26th day of illness was forwarded to Professor W. Schüffner, Director, Institute for Tropical Hygiene, Amsterdam, with the request that he would carry out agglutination tests with the various strains of *Leptospira* available in his laboratory. Professor Schüffner reported that no agglutination occurred with the twenty-one strains of *Leptospira* enumerated in Table I:—

TABLE I.

Strains of <i>Leptospira</i> .				Agglutination titre of patient's serum taken on 15th August, 1939.
Kantorowicz ⁽¹⁾	0
Hond. Utrecht IV ⁽²⁾	0
And. CH 31	0
Sejro M 84	0
Swart v. Tienen	0
Rachmat	0
Salinem	0
Hond. H.C.	0
Djasiman	0
Benjamin	0
Sentot	0
Sarmin	0
Vleermuis 90C	0
Naam	0
3705	0
Veldrat Bat. 46	0
Rat Semarang 173	0
Hebdomadis	0
And. CH 11	0
Ballico	0
Pomona	0

(1) 'Kantorowicz' is the classical strain.

(2) 'Hond. Utrecht IV' = *L. canicola*.

It was suggested to Professor Schüffner that, if the agglutination tests for leptospirosis were negative, he might try the mouse-protection test for yellow fever. This test could not be carried out in India where it is illegal to maintain virulent strains of yellow-fever virus.

(3) *Mouse-protection tests for yellow fever*.—The amount of the patient's serum available was insufficient to enable Professor Schüffner to carry out the mouse-protection test satisfactorily but, as he had obtained some evidence of yellow-fever immune substance, he asked for a further sample. Serum obtained from the patient on the 40th day after the onset of illness was sent and, on examination, it showed a positive 'Sawyer' test and an inconclusive 'Theiler' test. In reporting these findings Professor Schüffner stated: 'The interpretation is very difficult, and ought to be done with great caution. The positive

"Sawyer", together with the inconclusive "Theiler", point to the presence of protective bodies otherwise found only in men who have passed a yellow-fever infection. If our "Theiler test" had been completely positive, I would have decided without restriction for the diagnosis of yellow fever'.

Subsequently two specimens of the patient's serum obtained on 62nd and 116th days after the onset of illness were sent to Dr. G. M. Findlay, Wellcome Bureau of Scientific Research, London. Dr. Findlay's findings are summarized in Table II:—

TABLE II.

Particulars of sera.	Date of test.	Dilution of serum.	Number of mice out of six inoculated surviving test.	CONTROLS: NUMBER OF MICE OUT OF SIX SURVIVING TEST.	
				Known normal serum.	Known immune serum.
First specimen (62nd day)	... 23-10-39	1 in 8	5	0	5
" " "	... 2-11-39	1 in 32	6	0	5
Second specimen (116th day)	... 20-11-39	1 in 8	5	0	6
		1 in 16	5
		1 in 64	4

In reporting these findings Dr. Findlay stated: 'If the serum had come from an area where yellow fever is endemic, we should have no hesitation in saying that the patient had a recent attack of the disease, but naturally one hesitated to make such a statement about a case from India. That is the reason why we should so much like to hear the clinical history of the case'.

A specimen of the patient's serum taken on 387th day after the onset of illness was sent to Dr. J. H. Bauer, Rockefeller Institute, New York. Dr. Bauer reported that the serum possessed no protective action whatsoever against yellow-fever virus.

DISCUSSION.

In view of the consistently negative results of the animal inoculation and agglutination tests a diagnosis of leptospirosis in this case is not justifiable.

The results of the mouse-protection tests against yellow fever carried out with serum obtained from the patient between 26th and 116th days after the onset of illness are consistent with a diagnosis of yellow fever and there is little doubt that, had the patient been resident in an area of yellow-fever endemicity, a diagnosis of yellow fever would have been made. The complete absence of yellow-fever protective substance in the serum taken on 387th day after the onset of illness is not, however, in accordance with the usual findings in yellow fever in which protective substance is ordinarily demonstrable in the patient's serum for several years after an attack of the disease.

Sawyer, Bauer and Whitman (1937) have made a detailed analysis of the data relating to the specificity of the yellow-fever protection test. These workers record that out of 876 human sera obtained from Asia (China, Philippines, Java, Malaya, Ceylon and India) and from Australia where yellow fever has not been known to occur, only 3 specimens showed anomalous protective results. One was from a 13-year old child resident in Calcutta. This was tested thrice with a 10 per cent virus-suspension and gave protection once and an inconclusive result twice; another sample from the same child, obtained eight months later

and tested against a 20 per cent virus-suspension, was negative. Sawyer *et al.* (*loc. cit.*) classified this case as negative.

The two other sera from Asia which gave anomalous results were discovered among a batch of sera obtained from 87 adult Tamil males from the Madras Presidency, South India. The sera of two of the men, none of whom had ever visited a yellow-fever area, gave positive mouse-protection tests against a 10 per cent yellow-fever virus-suspension both with undiluted serum and with serum diluted 1 in 10. A second specimen of serum was obtained from each of these two men. The serum of one showed no protective substance when tested against a 20 per cent yellow-fever virus-suspension. The serum of the other, when tested against a 20 per cent suspension, was fully protective in dilution up to 1 in 10 but in a dilution of 1 in 16 inconclusive results were obtained and with a dilution of 1 in 32 no evidence of protection was observed. A third specimen was obtained from the latter individual about 3 years later; this protected against a 20 per cent suspension in a dilution of 1 in 2 but not in a dilution of 1 in 4.

Sawyer *et al.* (*loc. cit.*) also received anomalous results with the serum of a resident of Toronto, Canada. When tested against a 10 per cent yellow-fever virus-suspension, this serum (undiluted) gave protection on three occasions. A second specimen of serum from this individual taken 5 months later and tested against a 10 per cent suspension gave two positive, one inconclusive and one negative result. This serum was not tested against a 20 per cent suspension which, according to the originators of the test, gives much more clear-cut results.

Of the many hundreds of tests reviewed by Sawyer *et al.* (*loc. cit.*) only one (an adult Tamil male) gave a positive protection test and even in this case the results were inconclusive when the serum was diluted 1 in 16.

In the case described in this paper the patient's serum taken on 116th day after the onset of illness protected mice against a 20 per cent yellow-fever virus-suspension in a dilution of 1 in 64. No protective substance was, however, demonstrable by the 387th day after the onset of the disease.

SUMMARY AND CONCLUSION.

A Bengali girl, aged 11 years, who had been resident in Bengal all her life, developed a severe illness characterized by fever; severe headache; pains in the body, limbs and abdomen; nausea; persistent vomiting (blood-stained); hæmorrhages; intensive jaundice; and delirium. The acute attack lasted about 10 days, and after a protracted convalescence, the patient made a complete recovery. Elaborate laboratory tests failed to confirm a diagnosis of leptospirosis. Specimens of serum taken between 26th and 116th days after the onset of the disease showed the presence of yellow-fever immune substance but no trace of such protective antibodies could be found in a specimen of serum taken on 387th day.

The specificity of the mouse-protection test for yellow fever has been briefly reviewed in relation to these findings. The conclusion has been reached that the clinical features of the case and the serological findings indicate that the patient was suffering from a peculiar syndrome closely allied to yellow fever.

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REFERENCE.

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COMPLEMENT-FIXATION REACTION BETWEEN SERUM-PROTEIN AND ITS ANTIBODY FOR MEDICO-LEGAL PURPOSES; AND ASSOCIATED IMMUNOLOGICAL CONSIDERATIONS.

BY

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THE use of complement-fixation reaction has been suggested for detecting the source of blood for medico-legal purposes (Kolmer, 1923, 1929; Zinser, 1931; Lloyd, 1935). Although it is not likely to replace the precipitin reaction now universally employed for the purpose, it can help in making distinctions between type specific and group specific reactions which may not be possible with the latter reaction. Examples of strong group specific reactions in the precipitin reaction, are afforded by bloods of (1) sheep and goat, and (2) cow and buffalo. An antiserum prepared in fowls against sheep serum produces a ring of precipitate equally well with the goat or sheep serum, and an antiserum prepared against the goat serum also produces a ring of precipitate equally well with the sheep or goat serum. The same difficulty is encountered in dealing with the bloods of cow and buffalo. Even the distinction between the bloods of cow (or buffalo) and sheep (or goat) needs the use of specially selected antisera of equal potency and serial dilution of the extracts of material until one of the extracts fails to react.

With the antisera prepared in rabbits distinction between cow (or buffalo) and sheep (or goat) is easier and the one between cow and buffalo or between sheep and goat is possible. The possibility, however, is not always easily realized.

Complement-fixation succeeds, as a rule, in differentiating between the bloods of sheep and goat or cow and buffalo and makes a difference between sheep (or goat) and cow (or buffalo) much easier.

This communication deals with (i) a technique linked with the Wassermann reaction based on British methods, (ii) the reaction of antisera with sera from phylogenetically related animals, (iii) the application of the technique to medico-legal work, (iv) the difference between antibodies produced against the same serum-proteins but in different classes of animals, and (v) the categorically different reactions of complement-fixation.

I. A TECHNIQUE OF COMPLEMENT-FIXATION BETWEEN SHEEP SERUM-PROTEIN AND ITS ANTIBODY.

Immunological reagents.

1. *The hæmolytic system.*—This is the same as for the Wassermann reaction according to method No. 4 of the (British) Medical Research Committee (1918, now Council). The writers' choice of the complement is made in the following order: (1) Complement of 'optimal reaction and titre', (2) Complement of 'optimal titre', and (3) Complement of 'high titre' (Grevall, Chandra and Das, 1940). Complement of 'low titre' is rejected.

2. *The antigen*.—This is the normal sheep serum in a (1) constant 1 in 1,000 dilution in saline or (2) in serially weaker dilutions. In these dilutions it is not anti-complementary.

3. *The antibody*.—This is the antiserum produced in a rabbit. Intravenous injections of 2 c.c. of filtered and inactivated serum are given daily for 5 days. Five days after the last injection a sample of blood is taken from the ear vein. The serum (undiluted) from it should give a precipitin ring test as follows: (1) with a 1 in 1,000 dilution of the antigen a frank/sharp reaction in 2 minutes; (2) with a 1 in 20,000 dilution of the antigen a dubious reaction in 10 minutes and a frank/sharp reaction in 20 minutes; and (3) with a 1 in 40,000 dilution of the antigen a dubious reaction in 20 minutes. If the reaction does not come up to the standard another course of five intravenous injections is commenced.

If the reaction is up to the standard the rabbit is bled from the heart (not to death) 10 days after the last injection and the clear serum from the clot is inactivated and stored frozen in small bottles in lots of 0.2 c.c. It is thawed slowly 12 hours before use. Keeping the antigen constant the dilutions of the serum used are 1 in 100, 1 in 200 and 1 in 300. These dilutions are not anti-complementary. Alternatively keeping the strongest dilution of the serum (1 in 100) constant the dilutions of the antigen are weakened serially (1 in 10,000, 1 in 20,000).

Weak antisera with the limits of reaction fixed at 1,000, 5,000 and 10,000 (instead of at 1,000, 20,000 and 40,000) can also be employed. The dilutions used then are 1 in 25, 1 in 50 and 1 in 100 (instead of 1 in 100, 1 in 200 and 1 in 300). These dilutions should not be anti-complementary. Strong antisera are preferable and the dilutions in the ensemble are those of strong sera.

4. *The ensemble*.—

(1) *With constant dose of antigen*:—

Reagents.	FOR CONTROLS.		FOR TEST PROPER.		
	T u b e s		T u b e s		
	1	2	1	2	3
	(For antigen.)	(For antibody.)			
Antigen, 1 in 1,000 ...	1 vol.	—	1 vol.	1 vol.	1 vol.
Antibody, 1 vol. of, 1 in ...	—	100	100	200	300
Complement, 1 vol. containing ...	1 m.h.d.	1 m.h.d.	2 m.h.d.	2 m.h.d.	2 m.h.d.
Saline ...	1 vol.	1 vol.	—	—	—
Left at ice-box temperature 1 hour, at room temperature ½ hour and in incubator ½ hour.					
Sensitized r.b.c. suspension ...	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.

Incubated ½ hour.

Negative (—) and doubtful (±) reactions recorded at once.

Left at ice-box temperature overnight and positive (+) reaction or traces of lysis (T) recorded next morning.

(2) With constant dose of antiserum :—

Reagents.	FOR CONTROLS.		FOR TEST PROPER.		
	T u b e s		T u b e s		
	1	2	1	2	3
	(For antigen.)	(For antibody.)			
Antigen, 1 vol. of, 1 in ...	1,000	—	1,000	10,000	20,000
Antibody, 1 in 100 ...	—	1 vol.	1 vol.	1 vol.	1 vol.
Complement, in 1 vol. containing	1 m.h.d.	1 m.h.d.	2 m.h.d.	2 m.h.d.	2 m.h.d.
Saline ...	1 vol.	1 vol.	—	—	—
Left in ice-box, room and incubator as before.					
Sensitized r.b.c. suspension ...	1 vol.	1 vol.	1 vol.	1 vol.	1 vol.
Incubated $\frac{1}{2}$ hour.					
—, \pm , + and T reactions recorded as before.					

The second scheme does not give as clear-cut reactions as the first scheme. Incomplete fixation, however, is often observed even with the weakest dilution of the antigen.

Typical results are :—

	CONTROLS.		TEST PROPER.		
	Antigen.	Antibody.	T u b e s		
			1	2	3
With constant antigen ...	—	—	+	+	+/T/ \pm
With constant serum ...	—	—	+	+/T	T/ \pm

The reaction is the strongest in immunology. In both anaphylaxis [in which the weakest dilution of the reacting antigen may be of the order of 1 in 10 million in terms of serum-protein and 1 in 1 million in terms of serum (McIntosh, 1931)] and precipitin reaction (in which the dilution of the serum in the writers' laboratory is of the order of 1 in 40,000) the *antibody is used undiluted*. In the complement-fixation under description *both the antigen and the antibody are diluted*. Besides, the permissible limit of the strongest dilution of the antigen for the other two reactions is the same as in this reaction, namely. 1 in 1,000 dilution of the serum.

II. REACTION OF ANTISERA WITH SERA FROM PHYLOGENETICALLY RELATED ANIMALS.

In the first scheme of the ensemble, with constant dose of the antigen, are inserted the following additional tubes :—

In the controls, antigen from (i) goat and (ii) cow, i.e. one tube containing a 1 in 1,000 dilution of goat's normal serum and one tube containing a 1 in 1,000 dilution of cow's normal serum.

In the test proper 2 sets of 3 tubes each for goat's and cow's serum, i.e. one set like the sheep's serum set for goat's serum (in which there is goat's serum in the place of sheep's serum) and one set like the sheep's serum set for cow's serum (in which there is cow's serum in the place of sheep's serum).

Typical results according to the first scheme are:—

CONTROLS.				TEST PROPER.								
FOR ANTIGEN.			FOR ANTIBODY.									
T u b e s			Tube									
1 Sheep.	2 Goat.	3 Cow.	4 (Antisheep as before).	For sheep.			For goat.			For cow.		
				T u b e s			T u b e s			T u b e s		
				1	2	3	1	2	3	1	2	3
				+	+	+/T/±	+	+	T/±/-	+	+/-	-

There is *quantitative* difference (different grades of hæmolysis in a \pm reaction) between sheep and goat in the 2nd or 3rd tube and a *qualitative* difference (+ in one and — in the other) between sheep and cow in the 3rd tube.

The quantitative difference between goat and sheep also occurs when antigot serum is used in the test instead of the antisheep serum.

Similarly, the qualitative difference between cow and goat-sheep also occurs when anti-cow serum is used in the test instead of the antisheep serum.

The cow-buffalo relationship is of the same order as the sheep-goat relationship, with respect to themselves and other ruminants.

The results according to the second scheme, being less clear cut, are altogether quantitative.

The special feature of the technique is that the antiserum used is of a known precipitin titre, preferably a high titre. For antianimal sera a high titre is easily obtained. For antihuman sera, however, a high titre of this order is difficult to obtain.

III. APPLICATION OF THE TECHNIQUE TO MEDICO-LEGAL WORK.

PROBLEM: Is the stain caused by human blood or goat's blood?

Case 1. The precipitin test shows the presence of both human blood and also the blood of a ruminant animal (tested with an antisheep serum). The stain is, therefore, caused both by human blood and the blood of one of the following: (i) sheep and (ii) goat, constituting one sub-group, and (iii) cow and (iv) buffalo, constituting another sub-group, within the main group of ruminant animals. The reaction of the ruminant blood is strong, while that of the human blood though definite is not so strong.

Further precipitin tests with an antisheep serum and an antibuffalo serum prove the presence of the blood of (i) or (ii) only. The distinction between these two animals cannot be made with certainty except by the complement-fixation test.

The relative strength of the human serum and sheep-goat serum in the extract the total serum content of which is of the order of 1 in 1,000 (by 'foam test'; by comparison of its foam with that of a known 1 in 1,000 dilution) must next be determined. If the two reactions in the precipitin test were of the same strength, the strength of the two sera would be of the order of 1 in 2,000. The reaction of the human blood being not so strong as that of the

sheep-goat, the dilution of the two sera may be taken to be of the order of 1 in 2,500 and 1 in 1,500 respectively. Known controls of sheep and goat serum must, therefore, be 1 in 1,500. Greater accuracy is neither possible nor necessary. The significant differences in complement-fixation with varying quantities of the antigen are obtained with much larger gaps in dilution (1 in 1,000, 1 in 10,000 and 1 in 20,000, *vide supra*, second scheme).

The known sera in 1 in 1,500 dilution are put up side by side with the unknown serum (extract) as shown in the following ensemble:—

	Test with antiserum. Columns of tubes. (Each tube contains 1 vol. of a 1 in 1,500 dilution* of sheep, goat or unknown serum.)			Test with antigout serum. Columns of tubes. (Each tube contains 1 vol. of a 1 in 1,500 dilution* of sheep, goat or unknown serum.)		
	1	2	3	1	2	3
	Sheep.	Goat.	Unknown.	Sheep.	Goat.	Unknown.
1st row for 1 vol. of antiserum diluted 1 in	300	300	300	300	300	300
2nd row for 1 vol. of antiserum diluted 1 in	200	200	200	200	200	200
3rd row for 1 vol. of antiserum diluted 1 in	100	100	100	100	100	100

2 m.h.d. of complement and, later, r.b.c. suspension are added as before.

* The known dilutions should also be made equal by comparison of foam and further dilution of one of them if necessary.

Typical results may be:—

1st row	+/T	T	T	T	+/T	+/T
2nd row	+/T	T/±	T/±	T/±	+/T	+/T
3rd row	+	+	+	+	+	+

The reaction of the unknown serum corresponds to that of the goat serum in both the sets. The unknown serum is, therefore, goat serum.

If the reaction of the unknown serum corresponded to that of the sheep serum the unknown serum would be sheep serum.

Probably the animal blood has been added to mask the reaction of the human blood.

Case 2. The precipitin test shows the presence of the blood of a ruminant animal only. Further precipitin tests with antiserum and antibuffalo sera prove the presence of the blood of sheep or goat only.

From the stain make an extract corresponding to a 1 in 1,000 dilution of a serum and proceed as before with complement-fixation. The dilutions of the known sera will also be of the same strength.

IV. A DIFFERENCE BETWEEN ANTIBODIES PRODUCED AGAINST THE SAME SERUM-PROTEIN BUT IN DIFFERENT CLASSES OF ANIMALS.

The antisera for the precipitin test are prepared in the writers' laboratory from fowls (with the exception of the antifowl serum which is prepared from rabbits). Their titres are very high. They give with a 1 in 1,000 dilution of the appropriate serum a frank/sharp

ring within 2 minutes ; with a 1 in 20,000 dilution a dubious/frank ring in 10 minutes and a frank/sharp ring in 20 minutes ; and with a 1 in 40,000 dilution a dubious ring in 20 minutes. These potent sera fail to fix complement when used as antibodies in the technique described, i.e. an antibody made in a bird does not work with the complement derived from a mammal.

Conversely, an antibody made in a mammal does not work with a complement derived from a bird. This is shown by the failure of fresh normal fowl serum to hæmolyse sheep r.b.c. sensitized with sheep-rabbit hæmolytic amboceptor.

The antigen and antibody work regardless of the class of animals they are derived from. This is established by fowl-rabbit antiserum, used as antibody in the technique described, fixing complement with fowl's serum, used as antigen.

V. CATEGORICALLY DIFFERENT COMPLEMENT-FIXATION REACTIONS.

There are four categories of complement-fixation.

In the first category should be placed the reactions between the serum-proteins and their antibodies. The quantity of antigen needed is very small and that of antibody small. These reactions rank as the highest in immunology as has been stated before. They are specific.

In the second category should be placed the non-specific reactions of allergic states obtained in syphilis (W.R.), leprosy (Greval, Lowe and Bose, 1939) and kala-azar (Greval, Sen Gupta and Napier, 1939). These reactions are also strong. The quantity of the antigen used is, however, large and that of the antibody (which is really a reagin arising from allergic processes) not so small. Their diagnostic value is high.

In the third category should be placed the reaction between hydatid fluid and the patient's serum (Greval, Chandra and Das, 1941a, 1941b) and the reaction between a filtrate of rabies vaccine and its antiserum prepared in a sheep (Greval, 1933). They are of moderate strength and specific. Careful adjustment of the reagents is necessary.

In the fourth category should be placed the reactions of bacterial lysates or suspensions and the antiserum produced against the bacteria in the course of a disease (in man) or after inoculations [in animals, for producing antisera for therapeutic use (Greval and Dalal, 1933)]. These are specific but weak. Complement-fixation for gonorrhœa (Greval and Roy Chowdhury, 1945) belongs to this category. Very careful adjustment of the reagents is necessary.

To sum up : (1) the strongest reactions occur between serum-protein and its antiserum, (2) the second in intensity in allergic states, (3) the third in intensity between the fluids from an animal parasite in tissue and the patient's serum, and (4) the weakest between bacteria and the serum of the patient or the inoculated animal.

Of course all foreign proteins or protein products do not give rise to complement-fixing antibodies. Antitoxins which save life with almost mathematical precision are utterly inert. It is known that the antitoxins are contained in pseudoglobulins, while the anti-bacteria or antiviral bodies are contained in euglobulin (Fleming and Petrie, 1934).

Significance of a doubtful (T or \pm) complement-fixation reaction depends upon whether the reaction is specific or non-specific. For founding a diagnosis or opinion, with reference to the items mentioned in connection with the categories of complement-fixation and blood stains, a doubtful reaction will be rejected for syphilis, kala-azar and leprosy but accepted for other items, that is to say, the doubtful reaction of the second category cannot be considered reliable.

SUMMARY.

1. Complement-fixation reaction for determining the origin of blood in stains will not replace the precipitin reaction now universally used. It has, however, its place in distinguishing between the bloods of closely allied species when the latter reaction cannot be applied or fails.

2. A method based on the Wassermann reaction of the British technique has been described. The dilutions of the antibody used (specially prepared) are of the order of 1 in 25, 1 in 50, 1 in 100, 1 in 200 or 1 in 300, depending upon its precipitin titre. Only three of the dilutions are needed. The dilution of the antigen (in the stain) is of the order of 1 in 1,000 of the liquid serum. In these dilutions neither the antibody nor the antigen is anti-complementary. Two m.h.d. of the complement are left in contact with the antigen and the antibody for 1 hour in the ice-box, $\frac{1}{2}$ hour at room temperature and $\frac{1}{2}$ hour at blood heat. Sensitized r.b.c. suspension is then added and incubated in the usual way. The difference for cow (or buffalo) and sheep (or goat) is qualitative, and for cow and buffalo or sheep and goat quantitative.

3. The complement and the antibody must be derived from the same class of animals.

4. There are four categories in complement-fixation: (i) that between serum-protein and its antibody, the most sensitive and specific reaction in immunology, (ii) that between blood in allergic states and a heterologous antibody, like W.R., a strong but non-specific reaction, (iii) that between blood of an infested animal and extract from the infesting parasite a moderately strong and specific reaction, and (iv) that between blood of an infected animal and the infecting bacterium, a weak but specific reaction. Doubtful, \pm , reactions are significant only in specific reaction. Only antibacterial or antiviral bodies (as opposed to antitoxins) fix complement.

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PHARMACOLOGICAL ACTION OF AN ACTIVE CONSTITUENT ISOLATED FROM *VANDA ROXBURGHII* R.Br.

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INTRODUCTION.

Vanda roxburghii is an epiphytic herb of the N. O. *Orchidaceæ*. An indigene of the hotter parts of India, it is commonly known as 'Rasna' in Bengal and elsewhere. In the Ayurveda it is mentioned under the names Elaparni, Sugandha and various other names. Though clearly differing from the Ayurvedic Rasna in certain physical characteristics, *Vanda roxburghii* is said to possess many of the important therapeutic properties of that herb, among which mention may be made of its reported beneficial action in piles, rheumatism, sciatica and various other inflammatory and nervous diseases. The plant has been in extensive use both in the Hindu and the Yunani medicines; we, therefore, took up its pharmacological study, the results of which are noted below.

CHEMISTRY.

For the purpose of our investigation, the whole of the air-dried plant was used. Two kilograms of the powdered plant was thoroughly extracted with rectified spirit; the solvent was removed, and the residue taken up with water. The aqueous extract, after preliminary purification, was treated with lead acetate. The filtrate, after removal of lead, was neutralized, and then evaporated to dryness. The dry extract was next treated with alcohol, and then precipitated with ether. The precipitate being removed, the filtrate was evaporated to dryness. The residue was found to be glucosidal in nature, with well-defined physiological activity. This was further purified till the glucoside was obtained in a fairly pure condition. A fresh one per cent aqueous solution of this principle, the pH of which was adjusted to 6.4, that being the pH which gave fairly uniform results in preliminary experiments, was used in all the experiments mentioned hereafter.

Besides this active constituent, the plant also contains a bitter principle, tannin, resin, saponin, sterols, fatty oils and colouring matter.

PHARMACOLOGY.

Toxicity.—The material appeared to be non-toxic to frogs even in doses of 200 mg./kg. when injected into the ventral lymph sac. In rats and mice, it was found to be non-toxic when injected intravenously in doses of 150 mg./kg. and 300 mg./kg., respectively.

The alimentary system.—0.35 mg./kg. injected intravenously in the chloralosed cat, augmented the tone and movement of the intestines *in situ*. Atropine antagonized this action to a large extent, but not completely. Isolated pieces of cat's small intestines also showed augmentation of their tone and movements even with a dilution of 1 in 100,000. This augmentation was much reduced, but not completely abolished, by atropine 1 in 100,000 [Graph 2, figs. 4 (a) and (b)].

Respiration.—In the urethane cat, 0.50 mg./kg. to 1.0 mg./kg., given intravenously, increased the intra-tracheal pressure and the respiratory rate. Atropine antagonized these effects [Graph 1, figs. 3 (a) and (b)].

The cardio-vascular system.—

The heart: (1) *Mammalian heart in situ.*—Given intravenously, in the chloralosed cat, 1.0 mg./kg. slightly increased the systolic force of both the auricle and the ventricle. This systolic augmentation gradually disappeared, and the diastole finally became more pronounced. As the systole shortened, the ventricular rate gradually decreased. With a further dose of the same amount, the initial systolic and the later diastolic augmentations were observed as before; but the ventricular rate appeared to increase instead. After atropine, both the systolic and the diastolic augmentations persisted as before; but the decline in the ventricular rate was no longer in evidence.

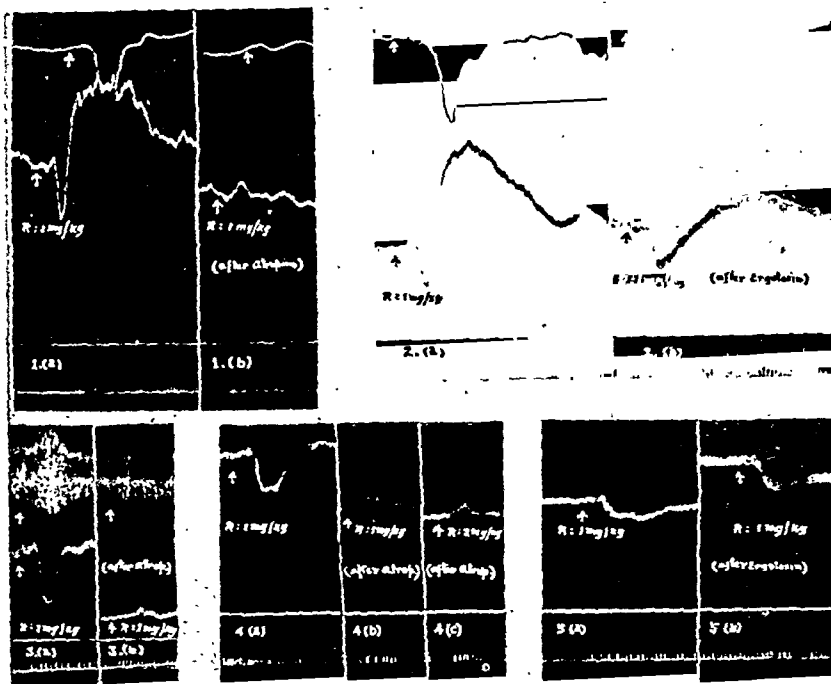
(2) *Isolated frog heart:* (a) *Force and rhythm.*—In a concentration of 1 in 3,000,000, the immediate effect of the drug was a slight augmentation of the systole without any change in the rate of the heart. This stage was followed by a stage of depression in which the systole and the rate both diminished, the rate eventually getting slower and slower. With increasing concentrations, these effects were more distinctly noticed. After atropine 1 in 15,000 the systolic augmentation was observed as before, but the rate continued unaffected.

(b) *Output.*—With the drug in a dilution of 1 in 3,000,000 no measurable alteration in the output was noticed immediately; a slight decrease, however, was observed later. With concentrations of 1 in 30,000 and higher, an initial increase in the output was noticeable, and this was followed a little later by a decrease. The return to normal output was delayed as the concentrations increased. After atropine 1 in 15,000, the initial increase in the output was observed as before; but the later decrease was not noticed [Graph 2, figs. 1 (a), (b), (c) and (d)].

The blood-vessels.—Perfusion with a dilution of 1 in 4,000,000 was followed by an increase in the outflow from the frog's systemic blood-vessels which decreased after a short time. Later on, it appeared to increase again and continued so for some time. With a dilution of 1 in 200,000, no immediate change was noticed; a slight increase was, however, observed after some time. This persisted for a short while and then gradually disappeared. The same effect was noticed with a dilution of 1 in 2,000 as well. After ergotoxin 1 in 1,000, the immediate effect observed was a slight increase in the outflow persisting for some time; after atropine 1 in 1,000, slight decrease was noticed instead.

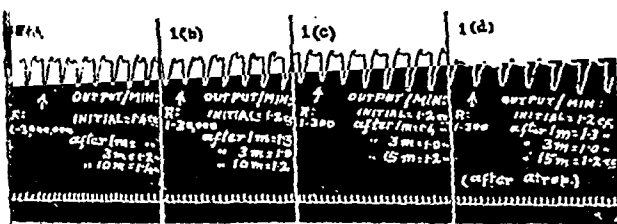
The carotid blood-pressure.—In the chloralosed cat, 0.35 mg./kg. to 1.0 mg./kg., given intravenously, invariably produced a fall in the carotid blood-pressure, followed by a rise to normal or beyond within a short time. With the higher doses, the fall was more abrupt, and the after-rise also was found to be higher, being in fact very high and abrupt with 1.0 mg./kg. An initial short rise, preceding the fall, was noticed in almost all instances. In the spinal cat, whose brain and cord had been destroyed, 1.0 mg./kg. to 2.0 mg./kg. intravenously was also followed by the same initial short rise and the succeeding fall; but there was no further after-rise beyond the original level. Atropine arrested the fall of pressure both in the intact and in the pithed animals; the subsequent rise, in these circumstances, was also absent; but the short rise preceding the fall remained unaffected. After ergotoxin, in both cases, the initial short rise and the succeeding fall were observed as before; but the after-rise, which in the pithed animal was hardly noticeable, was still found to be higher than the normal level in the intact animal although not to the same extent as before [Graph 1, figs. 1 (a) and (b), 2 (a) and (b), 5 (a) and (b) and 4 (a), (b) and (c)].

The spleen-volume.—0.30 mg./kg. to 1.0 mg./kg., given intravenously in the chloralosed cat, caused contraction of the spleen, synchronizing with the rise in pressure after the fall, and persisting as long as the pressure rose. Such contraction disappeared after both atropine and ergotoxin [Graph 1, figs. 1 (a) and (b) and 2 (a) and (b)].



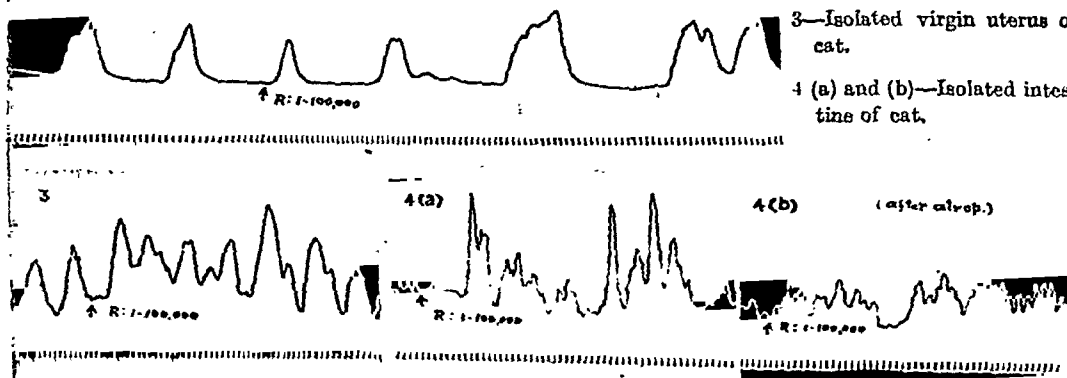
GRAPH 1.—Showing effect of *V. roxburghii* on:

- 1 (a) and (b)—B. P. and spleen volume before and after atropine.
- 2 (a) and (b)—The same before and after ergotoxin.
- 3 (a) and (b)—B. P. and respiration before and after atropine.
- 4 (a), (b) and (c)—B. P. in the spinal cat before and after atropine.
- 5 (a) and (b)—The same before and after ergotoxin.



GRAPH 2.—Showing effect on:

- 1 (a), (b), (c) and (d)—Isolated frog heart before and after atropine.
- 2—Isolated pregnant uterus of guinea-pig.
- 3—Isolated virgin uterus of cat.
- 4 (a) and (b)—Isolated intestines of cat.



The genito-urinary system.—

The urinary bladder : 0.35 mg./kg. increased the force of contraction of the bladder, but the frequency was diminished. After atropine, the movements diminished considerably, but were not abolished completely.

The uterus : (1) *Uterus in situ.*—The non-pregnant uterus of the chloralosed cat had increased tone and movement with doses of 1.0 mg./kg. intravenously. With 0.30 mg./kg. there was no distinct response. After atropine, the movements diminished, but were not completely abolished. (2) *Isolated uterus.*—With a dilution of 1 in 100,000, there was slight increase of tone and movement of the virgin uterus of the cat. Higher concentrations caused further increase. The pregnant uterus of the guinea-pig did not respond to any appreciable extent even with dilution of 1 in 50,000 (Graph 2, figs. 2 and 3).

DISCUSSION.

From the experimental results, it appears that the drug stimulates all organs having autonomic cholinergic nerve-supply. Atropine antagonizes this stimulation to a large extent, but does not completely abolish it. It seems, therefore, that apart from stimulating the cholinergic nerve-endings, the drug also exerts some direct action on the involuntary musculature of these organs.

The initial short rise of the carotid blood-pressure and the subsequent fall are both observed in the pithed cat; therefore, they could not be due to any central action. The former persists after atropine and ergotoxin; and appears to be due to the initial stimulation of the heart and the consequent increased output. The subsequent fall disappears after atropine, but not after ergotoxin; it therefore appears to result from stimulation of cholinergic nerve-endings. Cholinergic stimulation slows the heart and thereby causes diminished output from the organ; it also dilates the peripheral arterioles. The diminished output from the heart and the dilatation of the peripheral arterioles are together probably responsible for the fall of the carotid blood-pressure. The after-rise beyond the original level is not found in the pithed cat; therefore, it appears to be due to some central action. Such a rise is not to be found after atropine which abolishes the preceding fall; it is shortened, but not completely abolished, after ergotoxin. It seems, therefore, that this rise depends upon the preceding fall, and is partly adrenergic in character. As for the mechanism, it seems that the fall of the intra-carotid pressure results in stimulation of the vasoconstrictor centre, probably by reflex action through the carotid sinus, as suggested by Sollmann (1943); in response to this stimulation, all vessels with vasoconstrictor innervation divert a greater proportion of the blood supply to the weakly innervated cerebral and coronary arteries.

SUMMARY AND CONCLUSION.

1. An active constituent of glucosidic nature, which appears to be of very low toxicity in frogs, rats and mice, has been isolated from *Vanda roxburghii* in a fairly pure condition.
2. It has a stimulant action on organs innervated by cholinergic autonomic nerves, and to a small extent also directly on some involuntary muscles.
3. It lowers the carotid blood-pressure appreciably even in low doses.
4. Its effects appear to be due to (i) stimulation of cholinergic nerve-endings in the organs concerned, and later (ii) stimulation of vasoconstrictor nerves through the centre; they also appear to be due, to a small extent, to (iii) direct stimulation of involuntary muscles.

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THE LEVEL OF FAT INTAKE AND THE COMPOSITION OF SERUM LIPOIDS.

BY

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INTRODUCTION.

BURR AND BURR (1930) first observed that the blood serum of rats fed a fat-free diet absorbed less iodine than that of the control animals. Hansen and Burr (1933) later found that the concentrations of cholesterol and total fatty acids in the serum of rats fed a fat-free diet were lower than in the control animals and the iodine number of the serum fatty acids of the former indicated a higher state of saturation than that of controls. Williams and Maynard (1934) determined the total lipoids, phospholipoids, free and combined cholesterol and iodine number of the total lipoids in lactating goats receiving in one period a nearly fat-free ration and in the other the same ration supplemented with various oils. They found that the total lipoids and phospholipoids in the plasma dropped gradually on the fat-free diet and rose again on the other diets, irrespective of the type of fat used. In dogs the iodine number of the serum lipoids was found to be definitely lowered by low-fat diets by Hansen, Wilson and Williams (1936) and again in rats by Hansen and Brown (1937).

Hansen and Brown (1938) fed fats to rats at 20 per cent level. They found that there was a relationship between the level of the total serum lipoids and the degree of unsaturation of the dietary fats. The rats kept on the linseed oil diet had smaller quantities of total serum lipoids as compared with the animals on coco-nut oil. The cholesterol values tended to remain more constant, and the fatty acids of the serum appeared to maintain a rather uniform composition as to the degree of unsaturation present.

Von Gröer (1919, quoted by Brown, Hansen, Burr and McQuarrie, 1938) maintained two infants on a diet extremely low in fat over a period of several months. The growth of these infants was fairly good but both developed rickets due to the lack of fat soluble vitamin D in the special diet. Surprisingly enough one of them had an eczematous eruption of the skin because it occurred in spite of the regimen advocated in the treatment of eczema. After the resumption of a complete diet, both infants were found to be normal. Of 3 infants maintained on extremely low-fat diets by Holt, Tidwell, Kirk, Cross and Neale (1935) one developed marked eczema. The subject experienced a clinical remission when fat was added to the diet but again became eczematous, when the low-fat diet was resumed.

Hansen (1933a) found that the iodine value of the serum fatty acids in eczematous infants was less than that in the normal infants of comparable age. The same author (Hansen, 1933b) later reported that feeding of oils especially rich in unsaturated fatty acids brought about clinical improvement in cases of infantile eczema, which was accompanied by an increase to the normal value of the iodine number of serum fatty acids.

Brown *et al.* (*loc. cit.*) reported observations on two somewhat older infants kept on a diet extremely low in fat, though complete in other respects, for a period of ten weeks. The infants showed no signs of rickets and no skin manifestations except repeated mild attacks of impetigo. Analyses of their blood lipoids revealed a moderate decrease in the unsaturation of the serum fatty acids, similar to that reported by Hansen (1937) and by Faber and Roberts (1935) in infants with severe eczema.

Brown *et al.* (*loc. cit.*) determined the effects on a healthy man of a diet practically devoid of fat. A special fat-free diet supplemented with all the vitamins was fed for six months. A fall in the degree of unsaturation of the serum lipoids on a low-fat regimen was observed. The iodine value of the total fatty acids fell from 124 to 94 after six months. The subject showed no susceptibility to acute infections as a result of his extremely low fat intake.

Thus, in human beings as well as in other animals it has been shown that on experimental fat-free regimen the iodine number of the total fatty acids is lowered. Therefore, in groups of populations where fat intake is habitually low, certain deviations from the normal in the character of blood lipoids must be excepted.

So far no work is reported indicating any relationship of this kind. The present investigation was, therefore, undertaken to elucidate this particular point of view.

EXPERIMENTAL.

Blood samples were collected from healthy male subjects, between 20 and 40 years of age, from among the staff and students of the Seth G. S. Medical College, Bombay, and from the technicians and servant class as well.

The subject was asked to come in the morning with an empty stomach. Ten c.c. of venous blood were taken from the superficial cubital vein by a syringe. The blood was allowed to clot, centrifuged, and the clear serum taken for the analysis of total fatty acids, cholesterol and the iodine value of the total fatty acids. Bloor's method (1928) was followed for the estimation of total fatty acids and cholesterol. For the iodine value of fatty acids, Yasuda's method (1931) was adopted.

Table I gives the data regarding the average fat consumption and the values obtained for cholesterol, total fatty acids and the iodine value of the fatty acids.

The levels of fat intake in the various subjects were obtained by the diet survey method. Where this was not possible careful inquiries about the amounts of milk, ghee and oil purchased and consumed were made and the final figures obtained. The fat thus consumed is called the 'visible fat' which in the low-fat groups amounted to 10 g. to 12 g. per day per person, the rest of the fat was derived from cereals, pulses and other foodstuffs which again could be calculated from the respective quantities consumed. The figures for fat intake are not expected to be very accurate. They represent, however, the level of fat intake with a possibility of approximately 10 per cent variation from the values in column 2, Table I.

TABLE I.

Serum lipoids of human subjects with different dietary fat intakes.

Subject number.	Daily fat intake, g.	PER 100 C.C. SERUM.				Iodine number of fatty acids.
		Iodine absorbed, mg.	Total cholesterol, mg.	Total fatty acids, mg.	Total lipoids, mg.	
19	130	697	161	431	591	128
9	126	706	168	417	585	139
5	120	776	201	490	690	130
10	120	701	172	384	554	152
14	120	758	166	485	651	133
23	120	644	152	472	623	115

TABLE I—*contd.*

Subject number.	Daily fat intake, g.	PER 100 C.C. SERUM.				Iodine number of fatty acids.
		Iodine absorbed, mg.	Total cholesterol, mg.	Total fatty acids, mg.	Total lipoids, mg.	
12	110	706	173	532	705	110
15	110	635	161	433	593	121
21	110	763	182	385	566	164
24	110	529	187	381	568	105
26	110	785	177	468	645	139
7	100	741	175	509	685	122
13	100	683	171	404	575	132
17	100	741	155	398	553	159
18	100	776	158	484	641	141
29	100	816	175	492	667	141
66	100	794	175	400	575	169
44	90	537	174	395	569	111
80	90	582	130	420	550	117
82	90	653	199	340	532	152
85	90	728	177	454	631	134
81	85	645	113	461	574	123
6	80	706	204	485	689	117
11	80	653	167	393	560	137
45	80	557	162	359	520	125
83	80	582	174	413	587	112
76	76	595	183	239	422	195
84	75	582	159	400	559	118
35	75	578	157	396	553	119
8	70	715	163	447	611	135
16	70	675	167	401	568	140
39	70	582	155	348	503	137
60	70	512	150	355	505	116
67	70	619	150	358	508	144
68	70	602	156	343	499	142
69	70	688	167	374	541	153
73	65	662	130	475	605	121

TABLE I—*concl'd.*

Subject number.	Daily fat intake, g.	PER 100 C.C. SERUM.				Iodine number of fatty acids.
		Iodine absorbed, mg.	Total cholesterol, mg.	Total fatty acids, mg.	Total lipoids, mg.	
70	61	546	183	391	574	107
33	61	529	162	385	547	109
53	57	511	186	441	628	87
61	52	459	171	370	541	92
55	51	494	196	316	511	114
40	50	679	137	430	567	171
50	48	525	196	420	615	102
28	47	556	134	507	640	92
38	45	523	149	378	527	121
75	41	450	155	248	403	139
27	40	715	123	570	692	110
30	40	467	170	397	568	88
32	40	511	161	423	584	95
46	40	503	142	343	485	118
58	39	529	153	365	517	116
34	38	522	173	375	548	108
57	38	459	129	416	544	89
65	38	595	141	316	456	158
22	36	776	166	431	597	153
42	36	476	131	417	548	94
51	34	459	138	329	466	111
64	32	556	171	404	575	109
41	31	494	160	354	514	108
54	31	459	129	388	517	95
74	31	444	152	204	355	167
20	30	423	174	478	652	64
43	30	490	153	388	540	100
48	30	459	154	396	550	89
52	30	423	150	365	515	89
78	30	413	100	289	389	119
79	30	556	156	555	711	81
71	30	635	159	424	582	125
77	29	459	167	397	564	87
49	28	498	164	384	549	100
59	27	564	178	288	466	153
56	26	564	186	295	481	148
36	25	628	154	361	515	145

TABLE II.

Average values of serum lipoids.

Group number.	Range of fat intake, g.	Number of observations.	PER 100 C.C. SERUM.				IODINE NUMBER OF FATTY ACIDS.	
			Iodine absorbed, mg.	Total cholesterol, mg.	Total fatty acids, mg.	Total lipoids, mg.	Average.	Standard deviation.
I	91 to 130	17	721	171	445	616	135	18.1
II	61 to 90	22	616	163	392	555	130	22.4
III	31 to 60	23	529	155	384	539	115	25.7
IV	up to 30	12	509	158	385	543	108	29.5
A	61 and above	39	662	166	415	581	132	20.5
B	60 and below	35	522	156	384	540	112	25.9

DISCUSSION.

Cholesterol, total lipoids and total fatty acids.—The average values for cholesterol were a little higher in the group with the daily consumption of 100 g. to 130 g. fat, but in the remaining three groups the values showed very little deviation. Thus, within the limits investigated the dietary fat level seems to be without influence on the serum cholesterol values.

In the case of the average values for cholesterol, total lipid and the total fatty-acid concentration in serum statistically significant differences were not found to exist between the different groups. Even when the data were grouped in two classes, one having a daily fat intake above 60 g. and the other below that figure, no clear-cut conclusions could be drawn. All that can be said at the present moment is that further analyses should provide a larger sample which might possibly bring out the differences, the trends of which have become apparent in the figures presented here.

Iodine number of the fatty acids.—There appears to be a gradual decrease in the iodine values of fatty acids with the decrease in fat consumption. As the figures for the standard deviation show, the variation in any one group was rather large, and the differences in iodine number between the four groups (divided rather arbitrarily) were not statistically significant. When the data were put together in two larger groups, one having a daily fat intake above 60 g. and the other below 60 g. the average iodine values in the two groups were 132 and 112 respectively and the difference between the two was found to be statistically significant.

An attempt was then made to find out if there was any close association between the level of fat intake and iodine number of serum-fatty acids. Unfortunately, the figures obtained for the coefficient of correlation showed the absence of such association. One possible explanation for this is that in the diets of the subjects investigated, the level of fat could not have been the only difference between the different groups. If one bears in mind that the subjects belonged to different economic strata of society with monthly incomes ranging from Rs. 25 to Rs. 300 or more and that low fat intakes were commonly found in the lower income groups, it will be clear that there must have been other differences in the constitution and composition of the diet. Actually in the lower income groups the protective foodstuffs were consumed in extremely small quantities. It has also been observed that in

the lower income groups where the fat intake is low, a major portion (60 per cent) of the daily fat is derived chiefly from cereals, pulses and other articles of diet, a small quantity of oil used for cooking and a very small amount of milk used mainly in tea.

Then again the type of dietary fat mixture itself, which varies according to the quantity of milk products consumed, would be of some importance in determining the iodine number of serum-fatty acids (Hansen and Brown, 1938). Thus, it would appear that in addition to the level of total fat intake some other factor or factors might have possibly influenced the composition of the serum lipoids in the subjects under investigation.

Further work on the problem arising out of this investigation is in progress.

SUMMARY.

1. Analyses of total lipoids, total cholesterol, total fatty acids, and iodine absorbed in blood serum of 74 normal human subjects subsisting for long periods on different levels of dietary fat intake have been carried out. The values for fat intake were arrived at by diet survey methods.

2. The observations were grouped in two classes only, one containing individuals having daily fat intake above 60 g. and the other below this figure; the average figures for the iodine values of fatty acids differ significantly. The statistical treatment of the figures for the remaining constituents, however, does not give clear-cut indications about the significance in the different average values although they show a tendency to decrease with the decrease in fat intake. A larger sample is considered desirable.

3. The iodine value of fatty acids of serum and the level of dietary fat show a parallel decrease but no close association could be established between the two. It is suggested that in addition to the influence exerted by the level of dietary fat other differences in the composition of diets between the various groups might possibly have influenced the composition of serum fatty acids in the subjects investigated in the present series.

Dr. B. N. Acharya was associated with this work in its early stages as an I. R. F. A. worker; he gave great help in the standardization of the technique and analysed several samples of serum. The authors have great pleasure in thanking him for the valuable assistance rendered.

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STUDIES IN EXPERIMENTAL RICKETS.

THE CHANGES IN THE IONIC PRODUCTS OF CALCIUM PHOSPHATES AND THE SERUM ALKALINE PHOSPHATASE WITH THE ONSET, PROGRESS AND HEALING OF RICKETS.

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THE observations on clinical and experimental rickets made by Patwardhan, Chitre and Sukhatankar (1944, 1945) have shown that the onset, progress and healing of rickets are accompanied by changes in the ionic products of calcium phosphates in blood serum. The question naturally arose as to whether they were of such a magnitude as to be distinctly diagnostic of early vitamin D deficiency. Patwardhan (1943) had suggested that a comparative study of the changes in ionic products and serum alkaline phosphatase values might be made to decide which one of the two could give an earlier indication of vitamin D subnutrition. The present investigation was undertaken with this object in view.

EXPERIMENTAL.

Four young puppies from the same litter, four to five weeks old, were used for the experiment, the design of which was similar to the one described by Patwardhan *et al.* (1945). Two animals were given vitamin D deficient diets and the two controls received in addition a supplement of vitamin D. The diets used in this experiment are given in Table I:—

TABLE I.

Composition of rachitogenic diets.

Articles.		
	Diet I. Ca : P=1.33	Diet II. Ca : P=5.95
	g.	g.
Rice, milled	40.0	40.0
Chloroform extracted casein powder	2.5	1.25
White of egg equivalent of dried powder	1.0	2.8
* Salt mixture (Ca : P=2 : 1)	1.6	...
* Salt mixture P free	2.0

* The salt mixtures were prepared by slightly modifying Wesson's (1932) method in order to obtain the requisite Ca : P ratio on the diet as supplied to the animals.

The following supplements were administered directly in aqueous or oil solution, as the case required, in semi-weekly doses. Their quantities are given in terms of the dosage per day per dog :—

Thiamine hydrochloride	1 mg.*
Riboflavin	1 mg.
Nicotinic acid	10 mg.
Pyridoxine	1 mg.
Calcium pantothenate	1 mg.
Ascorbic acid	5 mg.
Vitamin A (diluted shark liver oil)	100 I.U./kg.*
Vitamin D (to control animals only)	100 I.U./kg.*
α -tocopherol acetate in oil	1 mg. per week.

It has been observed in this laboratory (Patwardhan *et al.*, 1945) that, irrespective of the Ca : P ratio in the diet, rickets could be induced in the young puppies provided the diet was deficient in vitamin D. The progress of the deficiency state, however, was rather slow in cases where the dietary Ca : P ratio was between 1 and 2, but with a high dietary Ca : P ratio, the process of induction of the rachitic state was considerably accelerated.

As the duration of the present experiment was estimated at about 10 weeks, it was decided to administer at first a diet with a normal Ca : P ratio and after 5 weeks a diet with a high Ca : P value. All animals therefore received diet I up to the 35th day when they were transferred to diet II.

The animals were bled from the external jugular vein once a week after overnight starving. Total calcium, inorganic phosphorus, and protein were estimated in the serum, using the methods described by Patwardhan *et al.* (1944). The alkaline phosphatase was estimated in the same sample of serum by the method of Giri and Shourie (1939). The relevant experimental results are illustrated in Graphs 1 to 4.

The x-ray pictures of the distal ends of radius and ulna were taken periodically to follow the progress of the deficiency state and its healing subsequent to vitamin D administration (Plates II and III, photos 1 to 20).

DISCUSSION.

Growth.—The growth of the animals was satisfactory until the change over to diet II. In 34 days on diet I the average increase in weight per dog was 1.24 kg. With the change of diet a retardation in growth was observed in three cases; only dog 27 kept on increasing in weight. The diminution in growth rate in the second period may probably be due to the drastic change in the dietary Ca : P value.

In an earlier experiment (unpublished work) it was observed that in the control animals showing rapid growth, vitamin needs were greater than in the slow growing animals particularly with regard to vitamins A, B₁ and D. Besides, it appeared that the greater the discrepancy between Ca and P values in the diet the greater was the need for vitamin D. In the present experiment the quantity of vitamin D administered to the control animals was 100 I.U. per kg. per day in the beginning; on change of diet it was soon apparent for reasons given later that this was insufficient and hence the vitamin D dosage of the control dogs 28 and 29 was increased to 200 I.U. per kg. per day. Dosages of some other vitamins were also doubled as has already been indicated.

Dog 28 died on the 67th day after showing signs of increasing weakness over a number of days. Post-mortem examination did not reveal any abnormality.

Calcium.—There was no marked change in the serum calcium content of either group until the diet was changed. After the dogs were put on diet II the serum calcium increased—as was to be expected on a low P diet—in both the experimental and in one of the control animals. In case of the other control animal (dog 29) the value increased enormously causing marked hypercalcaemia, at one time the maximum figure being 18.18 mg. per cent.

* The vitamin dosages marked with an asterisk were doubled on the 48th day of the experiment as there was reason to believe that the supply was insufficient.

PLATE II.

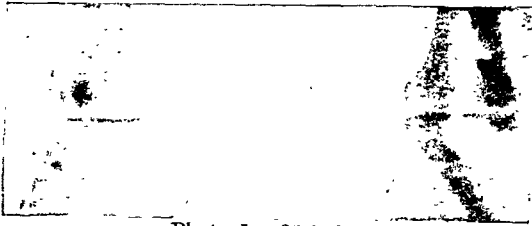


Photo 1. 26th day.



Photo 7. 26th day.

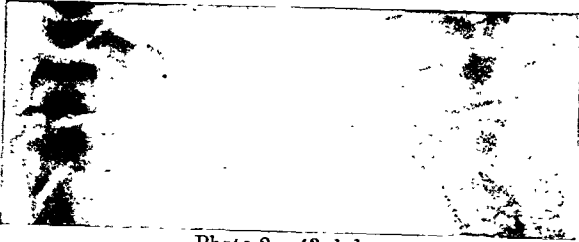


Photo 2. 43rd day.

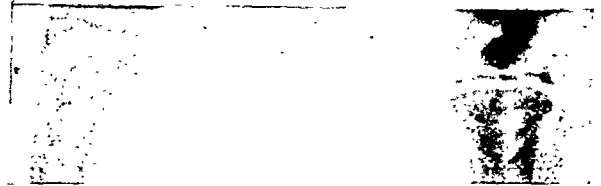


Photo 8. 43rd day.



Photo 3. 56th day.

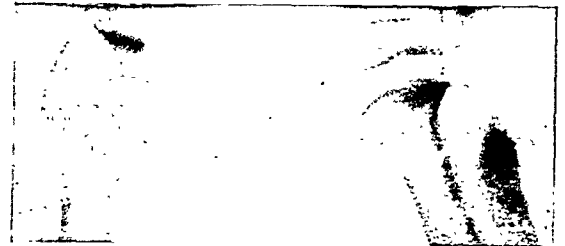


Photo 9. 56th day.

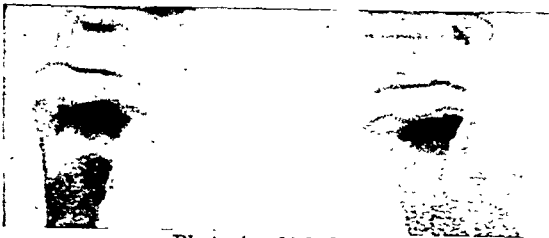


Photo 4. 64th day.

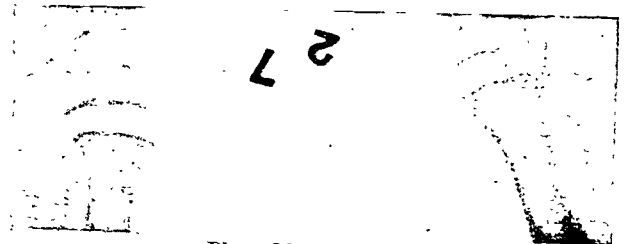


Photo 10. 64th day.



Photo 5. 71st day.

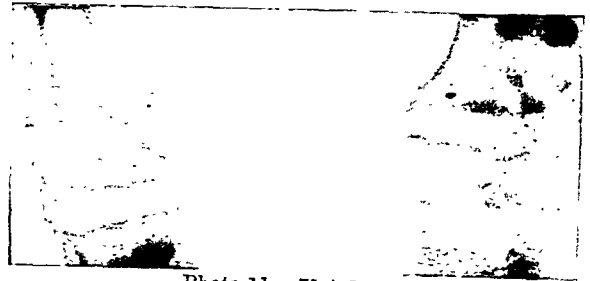


Photo 11. 71st day.

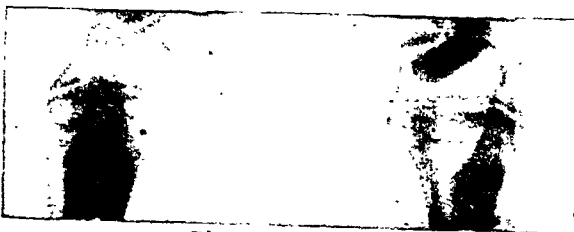


Photo 6. 78th day.

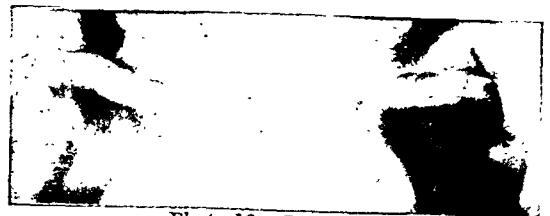


Photo 12. 78th day.

X-ray photographs of experimental animals, dog 26 on left and 27 on right; description in text.

PLATE III.

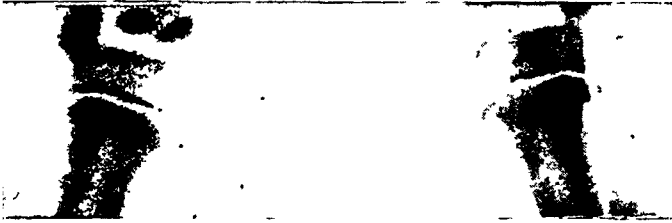


Photo 13. 25th day.

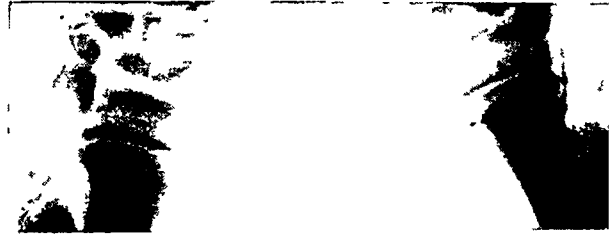


Photo 16. 25th day.

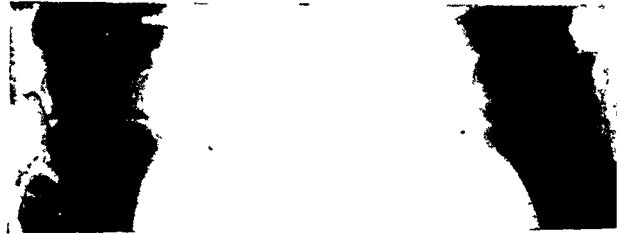


Photo 17. 42nd day.



Photo 14. 42nd day.



Photo 18. 55th day.

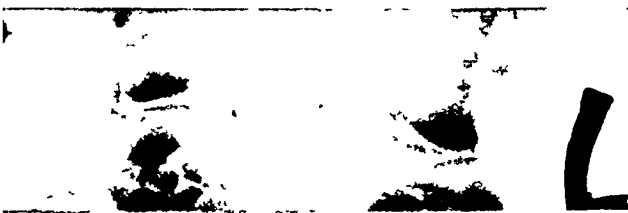


Photo 15. 57th day.

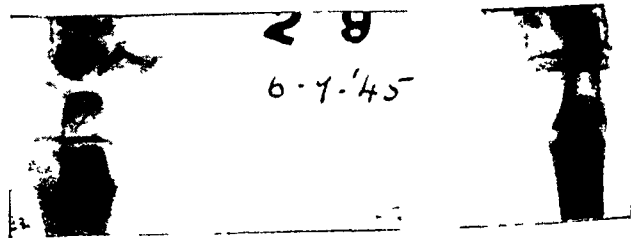
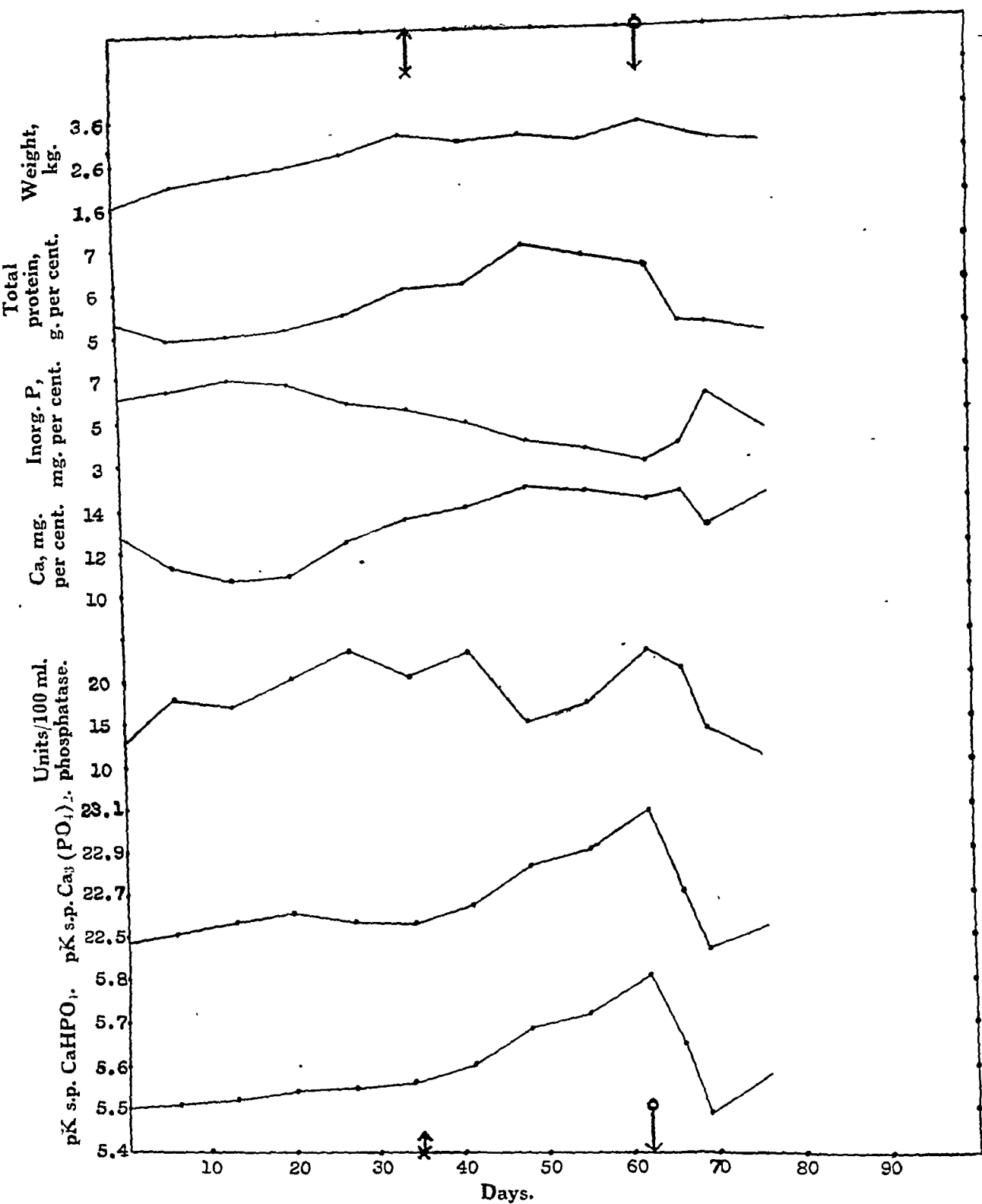


Photo 19. 70th day.



Photo 20. 77th day.

X-ray photographs of control animals, dog 28 on left and 29 on right; description in text,

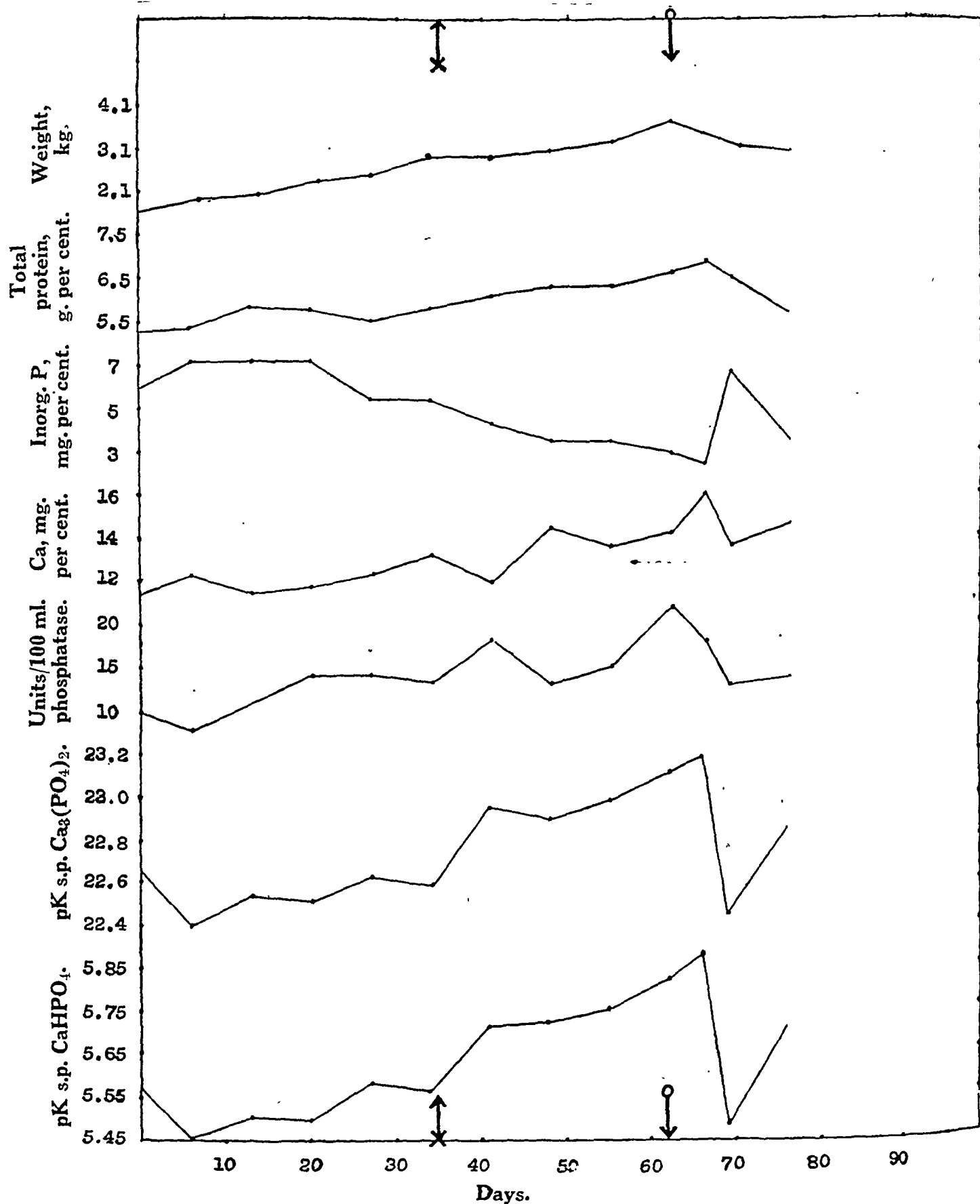


GRAPH 1.—Changes in concentration of some serum constituents in dog 26 kept on rachitogenic diet.

○
↓
↑
×

200,000 I.U. vitamin D in oil injected intravenously in a single dose.

Change from diet Ca:P = 1.33 to diet Ca:P = 5.95.

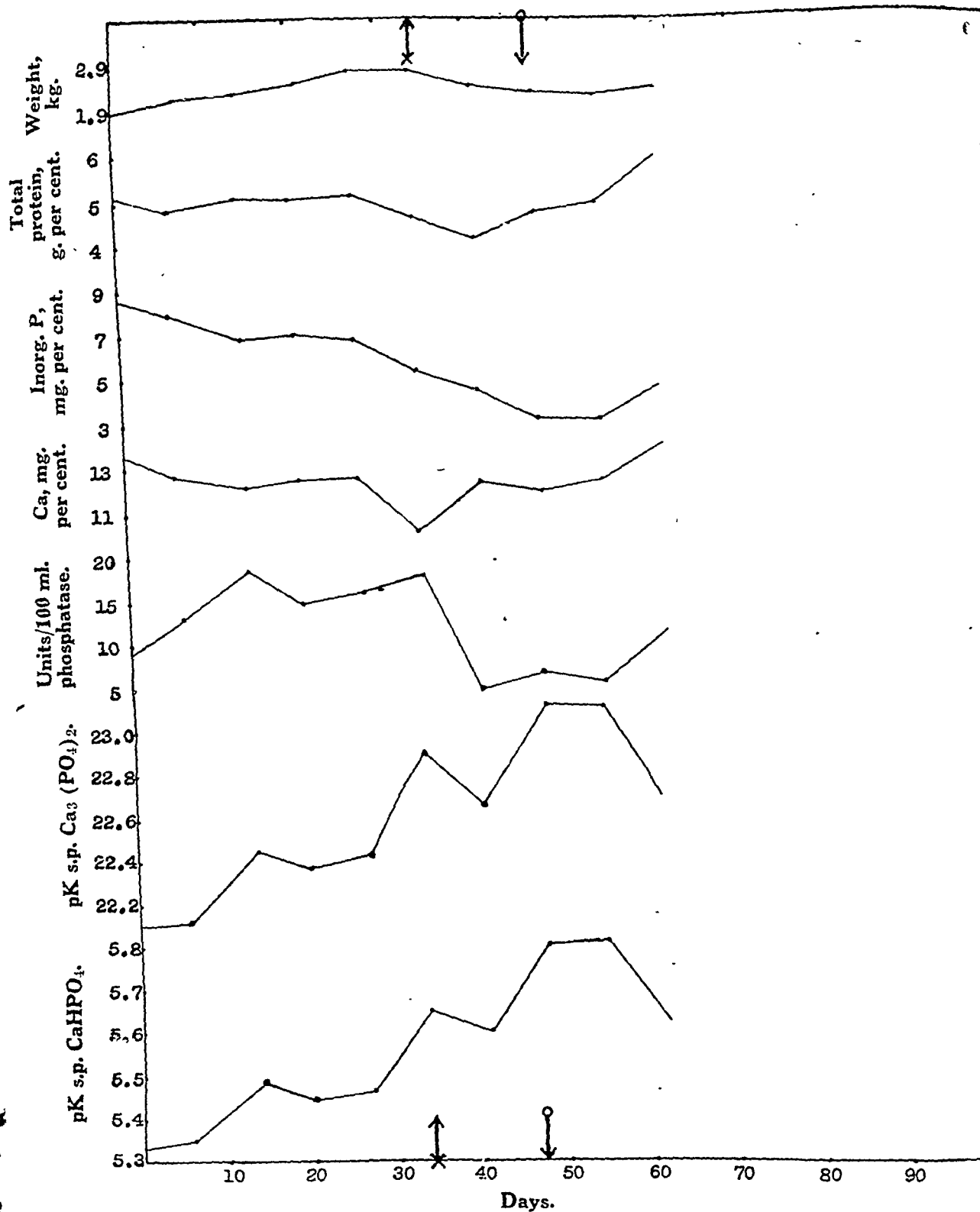


GRAPH 2.—Changes in concentration of some serum constituents in dog 27 kept on rachitogenic diet.

O
↓
↑
X

200,000 I.U. vitamin D in oil given orally in a single dose.

Change from diet $\text{Ca} : \text{P} = 1.33$ to diet $\text{Ca} : \text{P} = 5.95$.

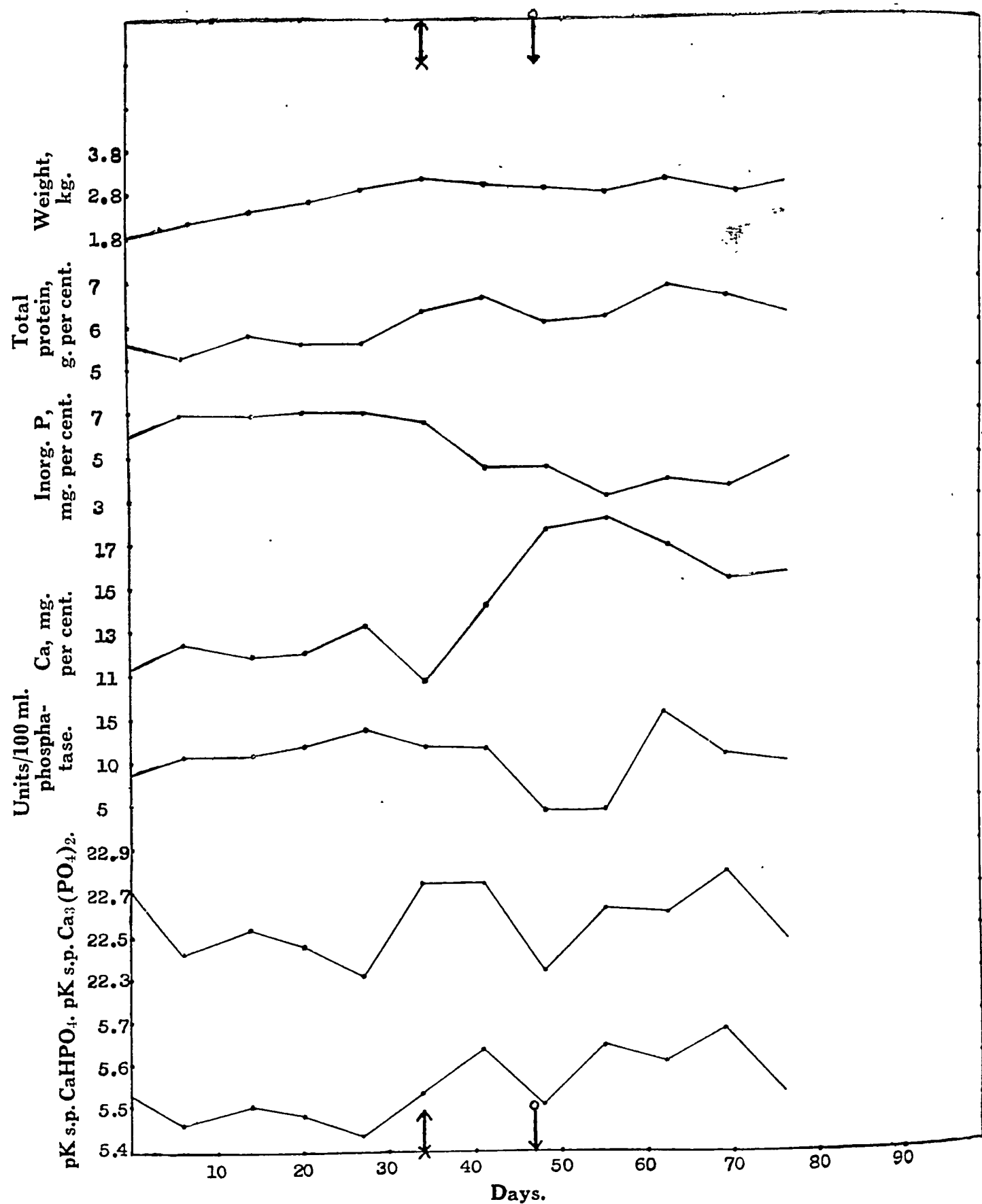


GRAPH 3.—Changes in concentration of some serum constituents in dog 28 kept on rachitogenic diet + supplement of vitamin D.

↓
↓
↑
↑
×

Vitamin D dosage doubled to 200 I.U./kg./day.

Change from diet Ca : P = 1:33 to diet Ca : P = 5:35.



GRAPH 4.—Changes in concentration of some serum constituents in dog 29 kept on rachitogenic diet + supplement of vitamin D.

O ↓ Vitamin D dosage doubled to 200 I.U./kg./day.

↑ X Change from diet Ca : P = 1.33 to diet Ca : P = 5.95.

On the 62nd day a single dose of 200,000 I.U. of vitamin D in oil was administered to both the experimental dogs, to dog 26 intravenously and to dog 27 orally. On the 4th day after the administration the serum calcium continued to increase but on the 7th day there was a marked fall in both the dogs which was synchronous with a rise in the serum inorganic phosphorus level. Again on the 14th day after the vitamin D dosage the level of calcium increased while that of inorganic phosphorus came down.

Inorganic phosphorus.—It will be clear from the figures that on diet I there was a slight but definite increase in the serum inorganic phosphorus at the beginning in three animals, whereas in dog 28 which had a rather high initial serum inorganic phosphorus value the downward tendency was marked from the beginning. After 20 days a decrease in the value was observed in both the experimental animals. Thus by the time diet II was begun the serum inorganic phosphorus in dogs 26, 27 and 28 had fallen from the initial values of 6.12 mg., 6.0 mg. and 8.62 mg. per cent to 5.42 mg., 5.39 mg. and 5.41 mg. per cent respectively.

The low phosphorus content of diet II caused an acceleration in the rate of fall of serum inorganic phosphorus even in the controls, a tendency which was checked by doubling the daily dosage of vitamin D as already mentioned (Graphs 3 and 4).

On the 62nd day the inorganic phosphorus values of dogs 26 and 27 were 2.98 mg. and 2.91 mg. per cent respectively. Within 7 days of the administration of the heavy dose of vitamin D, a sharp increase in the serum phosphorus value was noticed in both the experimental dogs. In dog 26 to which vitamin D had been given intravenously, a slight elevation was noticed as early as 4 days, whereas in dog 27 the rise in serum inorganic phosphorus occurred later.

Ionic products.—Patwardhan *et al.* (1944) have studied the ionic products of calcium phosphates in blood serum of rachitic and normal infants and children and have observed that the pK s.p. $\text{Ca}_3(\text{PO}_4)_2$ values above 23.0 were found in infants and children actually suffering from rickets or clinically suspected to be so, although the radiological evidence in the latter was negative or not available. Out of the 79 rachitic children and infants whom they studied, all but four had a pK s.p. CaHPO_4 value above 5.7. This may be taken to mean that a value higher than 5.7 could be associated with rickets.

Out of the two solubility products, viz. pK s.p. CaHPO_4 and pK s.p. $\text{Ca}_3(\text{PO}_4)_2$, the former appears to be a fairly sensitive index of the state of vitamin D deficiency once it is established. In the present experiment it was found that although the pK s.p. $\text{Ca}_3(\text{PO}_4)_2$ was still below 23.0 in both the experimental animals the radiological examination at the time testified to the onset of rickets (photos 2 and 8). This thus lends additional support to the observations of Patwardhan *et al.* (1945) referred to above on experimentally induced vitamin D deficiency.

As Graphs 1 to 4 indicate the solubility product of CaHPO_4 was showing a very slow increase in the animals on diet I. There was no increase in case of the control dog 29, but in the other control (28) the initial value of 5.33 was elevated to 5.65. The cause of this unexpected increase could not be satisfactorily explained since the radiological examination did not show any sign of rickets in the distal ends of the radius and ulna (Plate III, photos 13 and 14) of that particular animal.

The severity of the vitamin D deficient state in dogs 26 and 27 had become most marked radiologically by the 62nd day. A single massive dose of vitamin D then administered caused its effect to be felt within 7 days in both the experimental animals (in dog 26 within 4 days), as was determined by the pK s.p. CaHPO_4 values. In dog 27, however, there was a slight increase from 5.82 to 5.88 in the pK s.p. CaHPO_4 value on the 4th day, but the value on the 7th day was 5.48.

It is interesting to compare the values of serum Ca, inorganic P and the ionic products in dogs 26 and 27 (Graphs 1 and 2) on the 13th day after the administration of the heavy dose of vitamin D with photos 6 and 12 (Plate II). The latter give proof of fair progress towards the healing of the rachitic lesion, whereas the ionic products indicate a tendency to relapse to the deficiency state. The question naturally arises as to whether the single dose of 200,000 I.U. was sufficient or whether a further dose was necessary for continued healing. It would appear from Graphs 1 and 2 that a second dose of vitamin D would have been necessary

to prevent the relapse if the experiment had been continued further. This seems to require some explanation.

A mention has already been made that the intravenous administration of vitamin D brought on healing earlier than when it was given orally (Plate II, photos 5 and 11). On the basis of the studies of Morgan and Shimotori (1943) on the absorption of vitamin D from the gut it appears reasonably safe to conclude that the vitamin must have been almost completely absorbed from the intestine of dog 27 to whom it was given orally.

In both the animals, therefore, almost equally large amounts of vitamin D were available for use or storage. Polskin, Kramer and Sobel (1945) who have recently reviewed the evidence about the fate of vitamin D in the body suggest that the latter is inactivated or destroyed in some unknown manner, and that the storage of the vitamin accounts for a very small percentage of the total dose. It is likely that in the present experiment such a thing might have happened. Moreover, since the diet still continued to be rachitogenic the need for further vitamin D was reflected in the changes in serum Ca, inorganic P and the ionic products leading to a deficiency state. This possibly is the explanation of the 'kick back' apparent in Graphs 1 and 2.

Phosphatase.—It was Demuth (1925, quoted by Morris and Peden, 1937) who first reported high plasma phosphatase values in rickets. Later on, Kay (1932) observed a twenty-fold increase in the serum phosphatase values in rickets of different types, i.e. infantile, adolescent or renal. Bodansky and Jaffe (1934*a*, 1934*b*) confirmed these findings in infantile rickets but when they tried to correlate the increase in serum phosphatase with the intensity of rickets they failed to establish a satisfactory correlation in some of the rachitic cases, for in spite of an enlarged epiphyses and other clinical signs indicating active rickets the determinations of phosphatase indicated a completely normal state of health. These authors further observed that unless complete healing took place the serum phosphatase value remained at a slightly higher level than the normal.

Barnes and Carpenter (1937) have compared the reliability of the diagnostic procedures for rickets based on (i) the radiological findings, (ii) the fall in serum inorganic phosphorus and (iii) the increase in the plasma phosphatase. Their report on 187 cases indicates that whereas 25 per cent of the cases could be diagnosed radiologically and 20 per cent by serum inorganic phosphorus determination the estimation of serum phosphatase could help in the diagnosis of 65 per cent of the cases. Thus, it is clear that the phosphatase test gives a fairly reliable indication of the state of vitamin D deficiency.

Klasmer (1944) has reported a study of serum phosphatase activity in 1,000 children from 6 months to 1½ years of age and has come to the same conclusions as Barnes and Carpenter (*loc. cit.*). He observed, however, that only in 88 cases there was a close parallelism between the intensity of rickets and serum phosphatase activity.

The alkaline phosphatase of serum is affected by deficiencies of many other constituents of diets besides that of vitamin D (Freeman and Farmer, 1935; Weil and Russell, 1940; Gould and Schwachman, 1942; Bourne, 1943; Combs, Norris and Heuser, 1942; Dutra and McKibbin, 1945).

In clinical rickets there may occur concurrently several deficiencies besides that of vitamin D, for it is generally known that it is rare to come across cases of deficiency of only a single nutrient. In such cases then, the determination of serum phosphatase alone would not give a correct indication of the degree of uncomplicated vitamin D deficiency.

In the present experiment it was observed that, excepting in dog 26, the initial phosphatase values of all the animals were between 9 and 10 Bodansky units. The slightly elevated initial value of 13 units in dog 26 may lead one to suspect an incipient vitamin D deficient state of the animal. It may, however, be noted that in all the cases the phosphatase value gradually and steadily increased from the very outset. In dog 26 the maximum value was reached as early as the 27th day, the pK s.p. CaHPO_4 at that stage being 5.55. The radiological pictures of the animals on that day were equivocal (Plate II, photos 1 and 7).

A change in the Ca : P ratio of the diet from a low to a high value did not accelerate the increase in the serum phosphatase activity, inasmuch as excepting in dog 27 the value was very slightly changed. This observation might support the contention of Morris and Peden

(*loc. cit.*) that the serum phosphatase is not an altogether suitable index of the severity of vitamin D deficient state.

In the control animals (Graphs 3 and 4) a particularly interesting observation was that the change in the diet caused a sudden and unexpected fall in the phosphatase value which rose to its previous level after doubling the vitamin D dosage. The radiological examination of both the animals, however, did not show signs of rickets at any time.

The administration of a heavy dose of vitamin D to the experimental animals 26 and 27 caused in either case a marked fall within 7 days which was timed with a similar fall in the pK s.p. values of both the salts. It was found that whereas the solubility products had definitely reached the saturation level, the phosphatase value still lingered at a slightly higher level than the normal.

The radiological picture two days after the ingestion of vitamin D does not show any commencement of the healing but the x-ray picture taken on the 9th day reveals considerable progress in healing which is greater in dog 26 than in dog 27.

Thus, it will appear that the serum phosphatase increased as the vitamin D deficiency progressed. A change in the Ca : P ratio had, however, no appreciable influence on the serum phosphatase. When the changes in the serum phosphatase are compared with those in the pK s.p. values it will be found that the former gave a more correct picture of the state of vitamin D nutrition in the early, incipient and mild stages but (i) it could not provide a correct index of the severity of the disease caused by vitamin D deficiency and (ii) it could not indicate whether complete healing had taken place or not.

The pK s.p. figures on the other hand appear to be influenced by the dietary Ca : P ratio in addition to the influence exerted by the vitamin D deficiency, as is proved by the fact that they moved to the undersaturation level rapidly when the Ca : P ratio in the diet was increased. On administration of the vitamin D, the ionic products appeared to be affected earlier than the serum alkaline phosphatase and so a sudden fall in the value was noticed thus providing a surer index of the healing process.

SUMMARY.

Experiments were carried out on four healthy young puppies: (i) to study the time relationship between the increase in the serum alkaline phosphatase and of the solubility products of CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$ with the onset, progress and cure of the deficiency of vitamin D, and (ii) to determine which of the two would give a reliable indication of vitamin D deficiency in its early stages.

The animals were divided into two groups: control and experimental. Both the groups in the beginning received a diet containing a Ca : P ratio of 1.33, but after 34 days the latter was increased to 5.95. The animals received supplements of vitamin A, B complex, ascorbic acid and vitamin E and vitamin D (to control animals only) in adequate amounts. The experimental animals were given one dose of 200,000 I.U. of vitamin D on the 62nd day, the one orally and the other intravenously.

The animals were bled every week and serum analysed for calcium, inorganic phosphorus, phosphatase and total protein. Radiological pictures of the distal ends of radii and ulnæ were also taken. The findings are summarized below:—

The solubility products of CaHPO_4 and $\text{Ca}_3(\text{PO}_4)_2$ did not show an appreciable change towards the unsaturation level until the change over to the high Ca : P ratio diet. On changing the diet, however, the negative logarithm of solubility product increased rapidly with the progress of vitamin D deficiency in both the experimental animals; in the control animals the increase was not so rapid; moreover, it remained on the saturation side on doubling the vitamin D dose. Radiologically, also rickets was absent in the control. In the experimental animals the increase in pK s.p. CaHPO_4 appeared to be coincident with the increase in the severity of the disease and on the administration of the heavy dose of the vitamin D it suddenly decreased within 4 to 7 days: x-ray pictures on the 9th day indicated the commencement of the healing of bones.

In direct contrast to the solubility product, the serum alkaline phosphatase started increasing from the very beginning of the vitamin D deficiency and, on changing the diet,

the increase did not keep pace with the severity of rickets as judged by the x-ray pictures of the bones. Moreover, on the administration of the massive dose of vitamin D the value did not reach the normal rapidly but lingered at a slightly higher level than the normal.

The authors have great pleasure in thanking Messrs. Hoffman La Roche Inc. (U.S.A.) and Dr. T. J. Thompson Wells of Volkart Brothers (Bombay) for the free gift of pure vitamins used in these experiments. One of us (P. K. D.) is grateful to the Indian Research Fund Association for the grant of a Fellowship which enabled him to take part in this work. The authors also thank the Dean of the K. E. M. Hospital, Bombay, for permission to use the x-ray equipment and the staff of the department for taking the photographs.

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THE EFFECT OF DIFFERENT DIETS AND OF IRON MEDICATION ON THE NUTRITIONAL ANÆMIA OF INDIAN ARMY RECRUITS.

BY

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IN a previous communication (Hynes, Ishaq, Morris and Verma, 1946) we described the blood findings on eight hundred newly-joined South Indian army recruits, and demonstrated a correlation between the degree of anæmia and clinical signs of malnutrition. In this paper we report our observations on the response of these men to different diets and to iron medication during their first six months of training.

We compared three diets based on the Indian Army standard ration—the first unmodified, the second with a daily addition of eight ounces of meat (dressed weight), and the third a lacto-vegetarian diet in which the meat of the standard ration was replaced by tinned milk equivalent to 48 ounces of fresh milk daily (Table I). The two modified diets thus had an approximately equal animal-protein content. Full details of the composition of these diets, and of concomitant nutritional observations, will be reported separately by one of us (O. P. V.).

TABLE I.

*The actual average daily consumption (after allowing for wastage,
etc.) of recruits in the different diet groups during the
six months of the experiment.*

				DIET.
				Standard ration.
				Extra meat.
				Lacto- vegetarian.
Meat (dressed weight)	...	1.5 oz.	10 oz.	Nil.
Fish	...	1.5 oz.	Nil.	Nil.
Milk	...	6 oz.	6 oz.	54 oz.
Skimmed milk powder	...	$\frac{1}{2}$ oz.	Nil.	Nil.
Rice	13 oz.	
Atta (wheat flour)	7.5 oz.	
Dhal (pulses)	2.5 oz.	
Vegetables	10.5 oz.	
Fruit	1.5 oz.	
Sugar	3.25 oz.	
Ghee (cooking fat)	2.5 oz.	

TABLE I—*concl'd.*

				DIET.		
				Standard ration.	Extra meat.	Lacto-vegetarian.
Protein	{ animal	24 g.	46 g.	46 g.
	{ vegetable	79 g.	75 g.	74 g.
Carbohydrate	637 g.	617 g.	676 g.
Fat	93 g.	114 g.	129 g.
Iron	41 mg.	43 mg.	38 mg.
Calcium	827 mg.	625 mg.	2,078 mg.
Calories	3,800	4,000	4,300

We also tested the effect of ferrous sulphate at two dosage levels, three and six grains daily, given for the first four months of training.

MATERIAL AND METHODS.

This work was done between May and December 1945 in Harihar, Mysore State, at an altitude of 1,700 feet above sea-level. Except in May the weather was cool by Indian standards.

The subjects of the experiment were newly-joined South Indian recruits to a Pioneer Battalion. We described their racial composition and blood findings in our preliminary communication.

The men underwent normal training and the usual routine of vaccination and inoculation with T. A. B. and tetanus toxoid. They were not wormed.

Plan of experiment.—The experiment comprised seven treatment groups:—

A. IRON GROUPS (given 6 grains of ferrous sulphate daily for the first four months):

1. Standard ration diet.
2. Extra meat diet.
3. Lacto-vegetarian diet.

B. CONTROL GROUPS (no ferrous sulphate given):

4. Standard ration diet.
5. Extra meat diet.
- 6. Lacto-vegetarian diet.

C. HALF-IRON GROUP (given 3 grains of ferrous sulphate daily for the first four months):

7. Standard ration diet.

The composition of the different diets is shown in Table I.

Fifteen unselected men from the most recent arrivals at the Training Centre were taken into the experiment daily during May and June 1945.

In an experiment of this type all the characteristics which might affect the result should be evenly distributed throughout the treatment groups. In other words, any man taken into the experiment on any day must have equal chances of being assigned to any group. It is permissible to equalize the distribution of any one characteristic, but this must be done in such a way that the random distribution of both this and the other characteristics are not disturbed.

We chose to equalize the hæmoglobin distribution of our entrants. To each hæmoglobin level, at 0.5 g. intervals, we assigned a different set of the numbers 1 to 7 in a random order from published tables (Fisher and Yates, 1943). Each of these numbers represented a treatment group, and the order of the numbers was that in which men with a particular hæmoglobin level were to be distributed amongst the different groups. In this way the treatment groups were filled at nearly equal rates, and χ -squared tests showed that the frequencies of all the many characteristics observed were truly randomized over the groups. This random distribution was not disturbed by the heavy loss of subjects through sickness and absenteeism, but at the end of the experiment the numbers of subjects remaining in the different groups differed rather widely, but not significantly.

Each man was re-examined three times at eight-week intervals, so that for each subject we had four sets of data covering a total period of six months.

Technique.—The men were bled on all occasions at the same time of day in the early morning after breakfast. The techniques used were described in our preliminary communication (p. 119). At the end of the experiment we checked our hæmoglobinometer against an N. P. L. standardized Haldane hæmoglobinometer; there was no significant error.

We estimated the serum-protein level by van Slyke's copper-sulphate serum specific gravity method. Standard solutions were made from a stock containing 170 g. of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in 1,002.4 g. of water, and the serum-protein level was calculated from the formula:—

Serum-protein level (g. per 100 ml.) = (serum gravity — 1,007.2) \times 0.36.

Full details are given in a previous paper (Hynes, Ishaq and Morris, 1946).

Ferrous sulphate exsiccatus B. P. was suspended in water immediately before use and given just before the evening meal.

Statistical methods.—The principal statistic used to measure the rise in hæmoglobin level during the experiment was the hæmoglobin 'growth rate', that is the average rate of hæmoglobin increase. Graph 1, for example, shows the mean hæmoglobin levels at two-month intervals of the men of one of the treatment groups. The straight line drawn in the figure has been calculated so that it is the best possible representation of the trend of these four hæmoglobin levels. The slope of this line is the hæmoglobin 'growth rate'. It will be seen that the straight line over-simplifies the nature of the hæmoglobin increase—the hæmoglobin rose rapidly at the beginning of the experiment and fell slightly at the end. To study this progressive change in the hæmoglobin growth rate we use the curve drawn in the figure, and calculate the mean rate of change of the hæmoglobin 'growth rate'.

From each individual's set of four hæmoglobin readings we calculated, by the use of orthogonal polynomials (Fisher and Yates, *loc. cit.*), a curve of the form:—

$$\text{Hb} = A + Bt + C(t^2 - \frac{1}{2})$$

where Hb is the hæmoglobin level at the time 't', and 't' is measured in two-month units from the mid-point of the experiment. Thus, the linear regression function B is the mean hæmoglobin 'growth rate' during the experiment, and the term C is half the mean rate of change of the hæmoglobin 'growth rate'.

Analyses of covariance were made using these regression functions as dependent variates. The independent variates are defined in the appropriate sections below.

RESULTS.

There is always a possibility that illness will interfere with hæmoglobin regeneration, and some diseases, such as malaria, are certain to exert an adverse effect. We, therefore, excluded from our series all men who had, during the period of observation, been admitted to hospital with any disease other than 'common cold'. The latter term covered a group of mild febrile illnesses lasting for only two to five days.

TABLE II.

The mean hæmoglobin levels (g. per 100 ml.) on recruitment and after six months' training of men in the different groups. Ferrous sulphate was given daily for the first four months in the dose indicated.

Diet.	Dose of FeSO ₄ .	Number of cases.	INITIAL HB.		FINAL HB.	
			Mean.	S.D.	Mean.	S.D.
Standard ration ...	6 grains	75	14.47	1.4672	16.02	0.7591
	3 grains	84	14.50	1.4663	16.00	0.9494
	Nil	79	14.76	1.3538	15.79	0.9597
Extra meat ...	6 grains	91	14.68	1.4857	16.21	0.8219
	Nil	78	14.51	1.4093	15.86	1.0814
Lacto-vegetarian ...	6 grains	77	14.54	1.3375	15.94	0.7663
	Nil	85	14.70	1.3025	15.66	1.0523

The hæmoglobin changes in the different groups are summarized in Table II. It will be seen that there are considerable differences between the 'iron groups', whose members received ferrous sulphate for four months, and the 'control groups' whose members had no ferrous sulphate. There are smaller differences between the different diet groups. The significance of these differences is best tested by consideration of the hæmoglobin 'growth rates', that is the average hæmoglobin increase per two-month period of the different groups (Table III).

TABLE III.

The mean hæmoglobin 'growth rate' (g. per 100 ml. per two months) of the different treatment groups. The 'growth rate' is the linear regression function, and the adjusted values were obtained by the analysis of covariance.

DIET.							
Dose of FeSO ₄ .		STANDARD RATION.		EXTRA MEAT.		LACTO-VEGETARIAN.	
		Observed.	Adjusted.	Observed.	Adjusted.	Observed.	Adjusted.
6 grains	...	0.56 g.	0.53 g.	0.56 g.	0.57 g.	0.51 g.	0.50 g.
3 grains	...	0.54 g.	0.53 g.	—	—	—	—
Nil	...	0.40 g.	0.43 g.	0.47 g.	0.46 g.	0.37 g.	0.39 g.

We analysed the covariance of the linear regression functions, using the initial hæmoglobin level, the height increase in six months (as a measure of adolescence), and the hookworm load as independent variates. From the reduced sum of squares we have:—

	Degrees of freedom.	Sum of squares.	Mean square.	Variance ratio.	P.
Between groups ...	6	192.58	32.097	5.81	Under 0.001
Within groups ...	559	3,090.73	5.5290		
TOTAL	565	3,283.31			

(For this and similar tests of significance the 'between groups' sum of squares used is the difference between the 'total' and 'within groups' sums.) There are very clearly significant differences between the hæmoglobin 'growth rates' of the different groups, and the reduced residual mean square serves for 't'-tests on the individual adjusted means.

It may be mentioned that the analysis of covariance gave this experiment double the precision of a simple analysis of variance. The residual mean square of the analysis of variance of the growth rates is 13.3465, which is more than twice the reduced residual mean square of 5.5290.

TABLE IV.

The mean rate of change of hæmoglobin growth rate (g. per 100 ml. per two months) of the different treatment groups. This statistic is twice the term C of the best-fitting parabola, and the adjusted values were obtained by the analysis of covariance.

		DIET.					
		STANDARD RATION.		EXTRA MEAT.		LACTO-VEGETARIAN.	
Dose of FeSO ₄ .		Observed.	Adjusted.	Observed.	Adjusted.	Observed.	Adjusted.
6 grains	...	-0.52 g.	-0.50 g.	-0.46 g.	-0.47 g.	-0.46 g.	-0.47 g.
3 grains	...	-0.46 g.	-0.45 g.	—	—	—	—
Nil	...	-0.19 g.	-0.21 g.	-0.21 g.	-0.20 g.	-0.05 g.	-0.04 g.

Table IV shows the rate of change of the hæmoglobin growth rate in each group. (This is twice the term C of the best-fitting parabola to the data.) These statistics were submitted to the same analysis of covariance as the linear regression functions, and the reduced mean squares similarly lead to a very significant variance ratio. (The reduced residual mean square is 0.3297.)

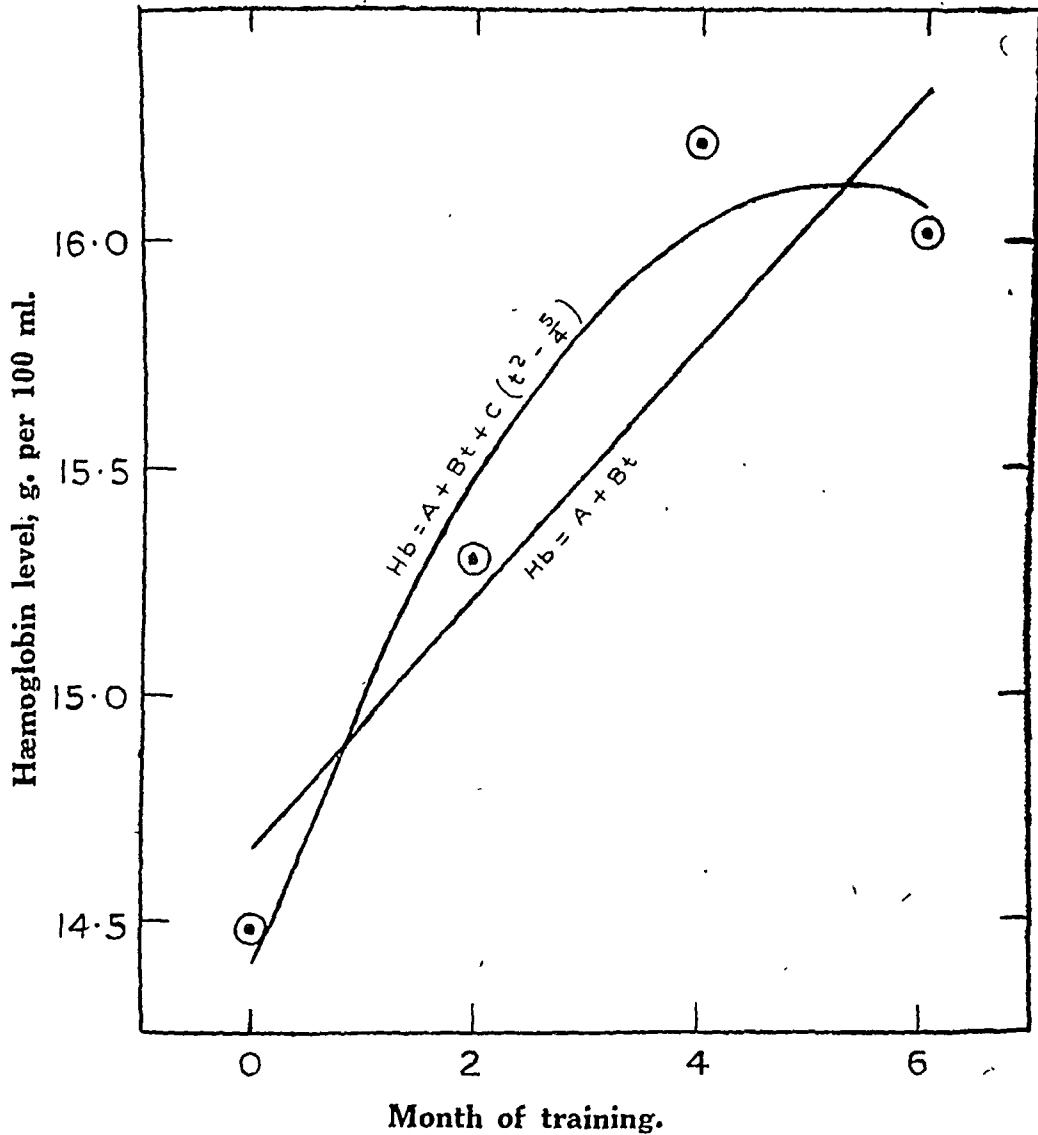
The effect of iron.—It will be seen from Table III that in all three diet groups men who received six grains of ferrous sulphate daily for the first four months of training had a substantially greater hæmoglobin growth rate than men not receiving iron. All three differences are statistically highly significant.

The differences shown in Table III are not numerically very large, but this is because the figures given are averages drawn from men with all grades of anæmia. If we consider the anæmic men separately then we see a much more dramatic difference, as illustrated by Graphs 2 and 3. Even the most anæmic classes of men receiving ferrous sulphate were restored to a normal hæmoglobin level in four months, whereas similar classes receiving the same diets but no ferrous sulphate were still anæmic at the end of six months.

A further point brought out by Graph 2 is that the hæmoglobin 'growth rate' of the iron groups was rapid for the first four months, but that during the last two months there was little change in the high level already reached. By comparison the hæmoglobin 'growth rate' in the control groups was slower and more constant. These facts are reflected

in the mean rates of change of the hæmoglobin 'growth rates' (Table IV); in all diet groups the 'iron group' has a negative value significantly greater than that of the control group.

The men of the standard ration group who received three grains of ferrous sulphate daily for the first four months of training improved just as rapidly as men who had had six grains daily. There was thus no advantage in the larger dose.

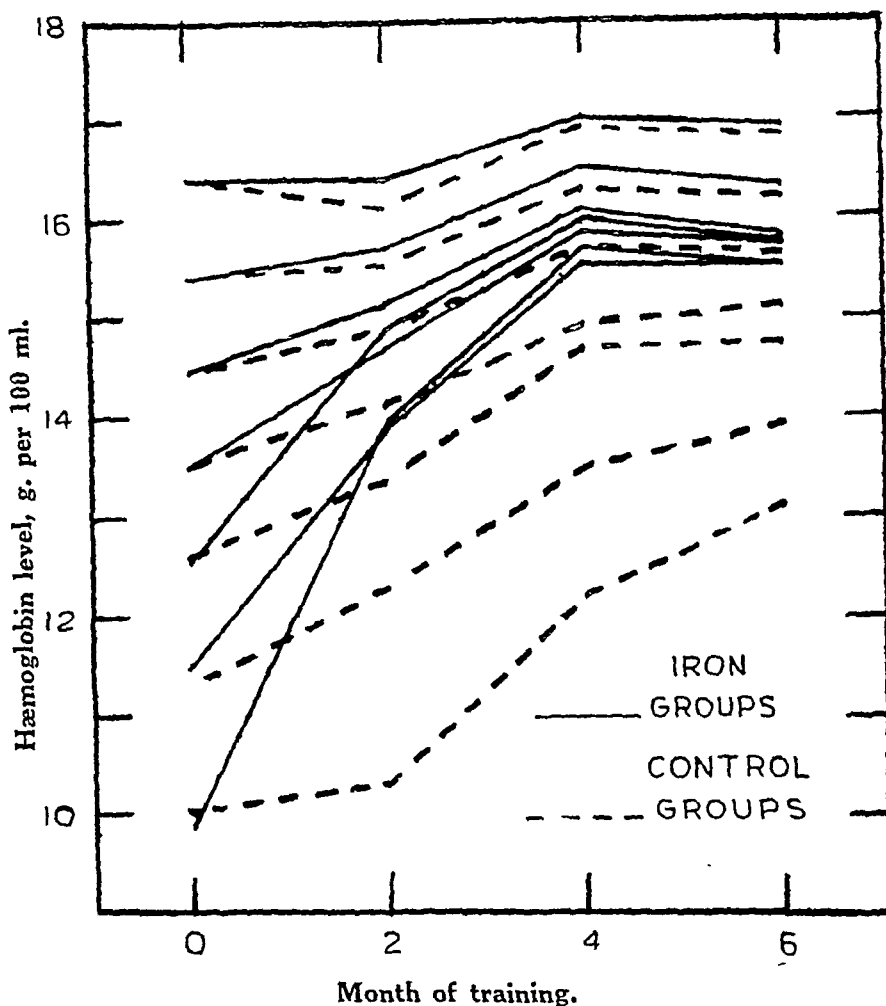


GRAPH 1.—The mean hæmoglobin levels on recruitment and at two-month intervals of men who took the standard ration diet and received six grains of ferrous sulphate daily for the first four months of training.

Some authors have found that iron medication raises the hæmoglobin to an artificially high level which is not maintained after the medication is stopped. We hoped by giving iron for only four months to obtain some evidence on this point, but it will be shown below that during the last two months there was a general fall in the higher hæmoglobin levels, equal whether ferrous sulphate had previously been given or not.

The effect of diet.—All three diets, whether ferrous sulphate was given or not, produced a substantial improvement in the anæmia of these recruits. Table III shows that the 'extra meat' diet appeared to have the greatest hæmatinic value, and the lacto-vegetarian diet the least; this difference was statistically significant.

The adjusted mean hæmoglobin 'growth rates' were compared by 't'-tests. In the 'iron groups' the mean 'growth rate' of the 'extra meat' group was not quite significantly greater than that of the lacto-vegetarian group ($P: 0.075$). The same relation held in the control groups ($P: 0.081$). If, however, we combine these two

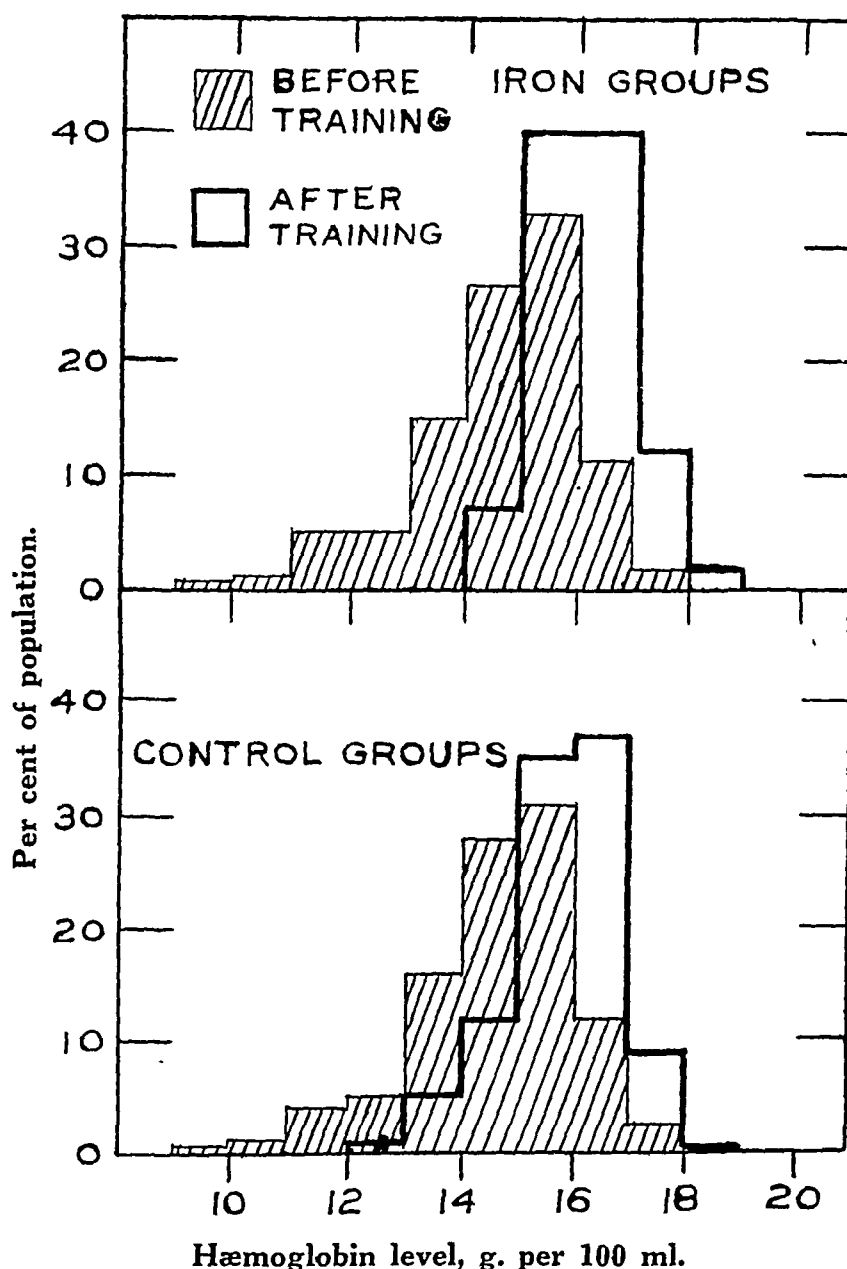


GRAPH 2.—The mean hæmoglobin levels at two-month intervals of recruits with different degrees of anæmia. The iron groups received 6 grains of ferrous sulphate daily for the first four months of training.

probabilities (Fisher, 1944, Sect. 21.1) we obtain a significant value of 0.04. The hæmoglobin 'growth rate' of the standard ration group was intermediate to the growth rates of the other two diet groups, but did not differ significantly from either.

There were no significant differences between the three diet groups in the rate of change of hæmoglobin 'growth rate'. It will, however, be seen from Table IV that the men of the lacto-vegetarian control group, who received no ferrous sulphate, had a very low negative rate of change of hæmoglobin growth rate. Their hæmoglobin level increased at a slow, nearly constant rate, whereas in other groups the greater part of the increase occurred in the first four months of the experimental period.

The hæmoglobin change of the last two months.—The hæmoglobin level, after rising steadily for the first four months of training, often fell slightly during the last two months, especially if a value over 16 g. had been reached. Table V shows the mean hæmoglobin change between four and six months of all men who had had no illness (other than common cold) during



GRAPH 3.—The hæmoglobin distribution on recruitment and after six months' training of men who received 6 grains of ferrous sulphate daily for the first four months of training (iron groups), and of men who had no ferrous sulphate (control groups).

their second three months of training. It will be seen that the 'iron groups' showed greater mean falls in hæmoglobin level than the control groups, but analysis showed that this was due purely to their higher four-month hæmoglobin level.

TABLE V.

The mean hæmoglobin change between the fourth and sixth months of training of men of the different treatment groups. The men of the 'iron groups' had received 6 grains of ferrous sulphate daily; the control groups had no ferrous sulphate.

Diet.	IRON GROUPS.			CONTROL GROUPS.		
	Number of cases.	Hb at 4th month.	Mean change 4-6 months.	Number of cases.	Hb at 4th month.	Mean change 4-6 months.
Standard ration ...	89	16.28 g.	-0.22 g.	86	15.84 g.	-0.12 g.
Extra meat ...	92	16.29 g.	-0.19 g.	92	15.63 g.	+0.15 g.
Lacto-vegetarian	83	16.15 g.	-0.23 g.	92	15.65 g.	+0.02 g.

Separate analyses of covariance were made for 'iron' and control groups, using the 4-6 months hæmoglobin change as dependent variate, and the 4-month hæmoglobin level and hookworm load as independent variates. In neither class was there any significant dietary effect, so the diet groups were thrown together to give two classes—'iron' and 'control'. There was no significant difference between these two (variance ratio 1.84, $P:0.18$). Finally, hookworm infestation was shown to be without effect.

We conclude that at the higher hæmoglobin levels (above 15.7 g. on the average) some physiological readjustment resulted in a slight fall in the hæmoglobin level between the fourth and sixth months of training, whether ferrous sulphate had previously been taken or not.

The effect of hookworm infestation.—Three-quarters of our subjects had hookworm infestation of some degree, but it was only in the more under-nourished third that infestation was correlated with anæmia (Hynes, Ishaq, Morris and Verma, *loc. cit.*). During the six months of the present experiment we found that infestation had no influence on hæmoglobin regeneration whether ferrous sulphate was given or not.

TABLE VI.

The mean hæmoglobin growth rates of men of the iron and control groups with different hookworm loads. The adjusted values were obtained by the analysis of covariance.

IRON GROUPS.						CONTROL GROUPS.				
Hookworm load.	Number of cases.	Initial Hb level.	HB GROWTH RATE.			Number of cases.	Initial Hb level.	HB GROWTH RATE.		
			Observed.	Adjusted.				Observed.	Adjusted.	
Nil	...	76	14.83 g.	0.47 g.	0.54 g.	56	14.69 g.	0.43 g.	0.45 g.	
Very light	...	114	14.57 g.	0.51 g.	0.51 g.	83	14.60 g.	0.43 g.	0.43 g.	
Light	...	52	14.40 g.	0.60 g.	0.56 g.	45	14.45 g.	0.41 g.	0.43 g.	
Moderate	...	65	14.40 g.	0.60 g.	0.57 g.	47	14.82 g.	0.38 g.	0.42 g.	
Heavy	...	20	14.27 g.	0.60 g.	0.53 g.	11	15.14 g.	0.32 g.	0.39 g.	
Reduced error mean square			5.246 g.	4.9291 g.				

In the main analysis of covariance the regression function of hæmoglobin 'growth rate' on hookworm load did not differ significantly from zero ('t': 0.196). This presumptive evidence that the hookworm load did not influence hæmoglobin regeneration was confirmed by analysis of the hæmoglobin 'growth rates' of recruits with different degrees of infestation. Analyses of covariance were made using the hæmoglobin 'growth rate' as dependent variate, and the initial hæmoglobin level, the height increase, and 'diet' as independent variates. Approximate allowance was made for the effects of the different diets by assigning the arbitrary values + 1, 0, and - 1 to the extra meat, standard ration, and lacto-vegetarian diets respectively. Separate analyses were made for the 'iron' and 'control' groups, but neither yielded a significant variance ratio.

In a similar fashion we showed that the rate of change of hæmoglobin 'growth rate' was not influenced by the hookworm load, whether ferrous sulphate was given or not.

The effect of intercurrent infections.—We were able, from our regular two-monthly blood counts, to assess the effect of intercurrent infections on hæmoglobin regeneration. Most of the subjects considered here were in hospital for less than three weeks, and we have excluded all who were in hospital for more than four weeks.

TABLE VII.

The mean hæmoglobin change (g. per 100 ml.) during the two-month period in which various infections occurred.

Disease.	IRON GROUPS.		CONTROL GROUPS.	
	Number of cases.	Hb change.	Number of cases.	Hb change.
'Common cold' ...	56	+ 0.88 g.	50	+ 0.47 g.
Malaria ...	17	+ 0.21 g.	18	- 0.28 g.
'I. A. T.'* ...	13	- 0.03 g.	17	- 0.16 g.
Acute tonsillitis ...	7	- 0.33 g.	1	+ 0.10 g.
Pneumonia ...	7	+ 0.93 g.	1	+ 1.10 g.
Dysentery ...	8	+ 0.85 g.	3	+ 0.03 g.
Childish fevers ...	11	+ 0.77 g.	8	+ 0.66 g.
Error mean square ...	1.4547		0.7496	

*Infection of the areolar tissues.

Table VII shows the mean hæmoglobin change in the two-month period in which various infections occurred. The figures for 'common cold' may be taken as a measure of the normal two-month hæmoglobin increase, for these mild infections, lasting only two to five days, may be assumed to have had little or no effect on hæmoglobin regeneration.

Separate analyses of the variance of these hæmoglobin changes were made for both 'iron' and control groups (both gave a significant variance ratio), so that for each disease two 't'-tests gave probabilities for the significance of differences from the 'common cold' means. These two probabilities were combined (Fisher, *loc. cit.*, Sect. 21.1) to measure the significance of the effect of the disease.

Malaria and 'I. A. T.' (infection of the areolar tissues) produced a highly significant depression of hæmoglobin regeneration, and acute tonsillitis produced a significant depression. The other diseases had no significant effect.

Malaria.—Malaria had a measurable depressant action on hæmoglobin regeneration, and in nearly half the cases a brief, promptly treated infection caused a fall in the hæmoglobin in place of the anticipated rise during the two-month period in which the infection occurred. Two cases may be cited to show how great may be the effect of a malarial infection which the Indian regards as trivial. Both men remained well during almost the whole six-month training period, but during the last two weeks they suffered from 'fever' every night. Neither reported sick nor asked to be excused his daily work. Their four hæmoglobin values, at two-month intervals, were:—

15.0 g., 14.3 g., 15.0 g. and 10.9 g. per 100 ml., and
15.6 g., 15.9 g., 15.5 g. and 12.7 g. per 100 ml.

In both the anæmia was normocytic and normochromic, and there was no bilirubinæmia. *P. vivax* were fairly numerous in the blood smears.

'I. A. T.'—'Infection of the areolar tissues' is an official Army diagnosis covering a range of infections from simple whitlow to chronic ulcer. These infections were usually almost healed within ten days, but they caused marked depression of hæmoglobin regeneration during the two months in which they occurred.

Acute tonsillitis.—This is not usually a severe or prolonged infection, but it too had a very definite depressant effect on hæmoglobin regeneration.

Long-term effects.—Intercurrent illness did not usually cause any depression of hæmoglobin regeneration, as measured by the hæmoglobin 'growth rate' over the whole six-month period. The one significant exception was that men who had had malaria and had not received ferrous sulphate had a low hæmoglobin 'growth rate' (0.17 g. per two months) compared with other groups. The men taking ferrous sulphate who had malaria had a normal hæmoglobin 'growth rate' of 0.55 g. per two months.

The effect of adolescence.—These recruits proved unable or unwilling to give their ages with even approximate accuracy, so that our best available method of distinguishing between adolescents and adults was the increase in height during the six months' observation. Using this criterion of age it appeared that the younger men were more anæmic than the older (Table VIII), and that anæmia in adolescents responded less completely to treatment than in adults.

TABLE VIII.

The hæmoglobin distribution on recruitment of recruits classified according to their height gain in the subsequent six months.

Height gained in 6 months, inches.	INITIAL HÆMOGLOBIN LEVEL, G./100 ML.										Number of cases.	Mean.	S.D.
	8—	9—	10—	11—	12—	13—	14—	15—	16—	17—			
<i>Nil</i>	1	5	9	15	33	60	30	3	156	14.91	1.3980
0.25	...	1	3	4	6	27	50	57	16	5	169	14.67	1.3765
0.5	...	1	1	6	5	21	34	34	14	4	120	14.56	1.4778
0.75	4	5	17	24	17	7	...	75	14.28	1.3791
1.0	1	2	5	11	5	1	...	25	14.18	1.1180
1.25	1	3	...	4	6	3	1	...	18	13.79	1.6450
1.5	1	3	2	6	13.48	...

The main analysis of covariance gave a significant negative regression function for hæmoglobin 'growth rate' on height increase ($t: 4.637$), that is the more height a man gained, the less, relatively, was his gain in hæmoglobin. The analysis of this effect was complicated by a strong negative correlation between initial hæmoglobin level and the subsequent height increase. The analysis of variance proved that the differences in initial hæmoglobin level in different height-increase groups were highly significant (the residual mean square was 1.9030 g.). Judging by fluctuations in the heights of individuals measured at two-month intervals, a height gain of 0.25 inches may be only postural, but greater gains in height must be regarded as genuine growth, and were associated with significantly low values for the hæmoglobin level on recruitment.

OTHER MEASUREMENTS.

All types of anæmia appeared to respond equally well, and as the hæmoglobin level rose the various red cell measure moved towards the 'normal' values of men with 15 g. hæmoglobin or over.

Red cell counts.—The red cell count is of course strongly correlated with the hæmoglobin level, but Table IX shows that at equivalent hæmoglobin levels there was no significant difference in the red cell counts of 'iron' and control groups.

TABLE IX.

The distribution of the red cell count and hæmoglobin level in recruits after six months' service.

Hb level.	Group.	RED CELL COUNT — M/CU.MM.						Number of cases.	Mean.	S.D.
		4.0—	4.5—	5.0—	5.5—	6.0—	6.5—			
12 g.	Control	1	2	3	5.00	...
	Iron
13 g.	Control	...	2	5	4	1	...	12	4.99	0.2347
	Iron
14 g.	Control	7	19	4	...	30	5.14	0.2566
	Iron	...	1	13	11	1	...	26	4.93	0.2911
15 g.	Control	17	61	7	...	85	5.14	0.2332
	Iron	27	86	12	...	125	5.12	0.2211
16 g.	Control	5	48	36	1	90	5.40	0.2648
	Iron	9	83	34	1	127	5.31	0.2449
17 g.	Control	7	11	3	21	5.61	0.2816
	Iron	12	23	9	44	5.66	0.3128
18 g.	Control
	Iron	1	2	4	6.40	...

Mean corpuscular volume.—Table X shows the MCV (mean corpuscular volume) distribution of recruits of the 'iron' and control groups. Analyses of variance showed that in the 'iron' groups the MCV at the end of the experiment did not vary significantly with the hæmoglobin level, but in the control groups the MCV was very significantly subnormal in men with less than 15 g. hæmoglobin per 100 ml.

TABLE X.

The distribution of the mean corpuscular volume and hæmoglobin level in recruits after six months' service.

Hb level.	Group.	MEAN CORPUSCULAR VOLUME.					Number of cases.	Mean.	S.D.
		80—	85—	90—	95—	100—			
12 g.	Control	...	1	2	3	84.67	...
	Iron
13 g.	Control	6	4	2	12	89.50	3.4245
	Iron
14 g.	Control	...	3	11	11	3	29	89.45	4.4286
	Iron	...	1	9	10	4	24	90.50	3.8785
15 g.	Control	...	2	15	33	21	71	92.11	3.9226
	Iron	...	1	30	34	21	90	91.89	4.0709
16 g. & over	Control	...	1	10	21	13	48	92.94	4.5961
	Iron	...	4	20	34	28	89	92.49	4.2456

Mean corpuscular hæmoglobin concentration.—The MCHC distributions in the 'iron' and control groups at the end of the experiment are shown in Table XI. In the 'iron' groups the MCHC did not vary significantly with the hæmoglobin level, but in the control groups hæmoglobin values below 15 g. were associated with very significantly low MCHC's.

TABLE XI.

The distribution of the mean corpuscular hæmoglobin concentration and hæmoglobin level in recruits after six months' service.

Hb level.	Group.	MCHC PER CENT.						Number of cases.	Mean.	S.D.
		26—	28—	30—	32—	34—	36—			
12 g.	Control	...	1	1	1	3	29.17	...
	Iron
13 g.	Control	2	4	5	...	12	31.08	1.7299
	Iron
14 g.	Control	3	12	13	...	29	31.81	1.3655
	Iron	9	11	3	24	32.79	1.3345
15 g.	Control	19	45	7	71	32.67	0.9708
	Iron	17	58	15	90	32.99	1.0731
16 g. & over	Control	7	35	6	48	33.02	1.0313
	Iron	8	64	15	89	33.30	1.1688

Serum-protein level.—At the end of the experiment there were no significant differences in the serum-protein levels of the different treatment groups, and the serum-protein level was not significantly correlated with the hæmoglobin level ('r' = +0.057, 't': 1.360, P: 0.17).

We have shown elsewhere (Hynes, Ishaq and Morris, *loc. cit.*) that the mean serum-protein level of recruits is above that of trained soldiers. Table XII shows the serum-protein level distribution of our recruits after six months' training compared with that of trained men of the same unit with over one year's service. The recruits' mean did not differ from that of trained soldiers, but the recruits' values were spread over a wider range. That this is a significant difference is proved by comparing the two standard deviations ('z': 0.2792, 0.1 per cent value 0.2685).

TABLE XII.

The distribution per cent of the serum-protein level in recruits after six months' service and in trained soldiers.

(Calculated from the serum specific gravity.)

Serum specific gravity.	Serum proteins, g./100 ml.	Recruits.	Trained soldiers.
1,023	5.7	0.5	...
1,023.5	5.9
1,024	6.1	1	...
1,024.5	6.2	2	1
1,025	6.4	9	9
1,025.5	6.6	12	11
1,026	6.8	22	30
1,026.5	7.0	20	26
1,027	7.1	18	16
1,027.5	7.3	7	6.5
1,028	7.5	6	...
1,028.5	7.7	2	1
1,029	7.8	0.5	...
<hr/>			
Number of cases	...	569	90
Mean	{ gravity	...	1,026.35
	{ protein	...	6.89
S.D.	{ gravity	...	0.9569
	{ protein	...	0.3445

DISCUSSION.

There is little doubt that malnutrition is the primary cause of the anæmia of the poor Indian. The anæmia is commonly ascribed to the outstanding deficiency of protein, and especially of animal protein. Even a light hookworm infestation will appreciably add to the anæmia of an under-nourished man, and malnutrition and multiple vitamin deficiencies lower his resistance to infections which still further depress his hæmoglobin level. Finally, malaria is a most potent cause of anæmia whenever the Indian leaves his village and meets

new strains of the parasite, and probably even in his home chronic malaria contributes to his anæmia.

This experiment has shown that the recruits' anæmia is cured by an adequate diet quickly if a little ferrous sulphate is given for a few weeks, more slowly if it is not. Our data supply no evidence as to what fraction or fractions of the Army diet cure the anæmia, but the relatively high protein content must be of great importance. Whipple, by his experiments on dogs, has shown that hæmoglobin synthesis demands an adequate supply of protein as well as of iron.

We showed in our preliminary communication that the anæmia of these recruits was nearly always associated with hæmatological signs of iron deficiency, so that to cure the anæmia a diet must supply enough iron not only to form some two or three hundred grammes of new hæmoglobin, but also to correct a long-standing iron deficiency. The Army diet does not supply enough iron for these purposes in six months, and the iron deficiency may persist even longer. For example, we found three cases of mild iron-deficiency anæmia amongst 90 unselected junior Indian N.C.O.'s who had worked for nearly two years in the Training Centre, where the diet is uniformly good and the standard of hygiene high.

Even the more severe grades of anæmia were completely cured by a daily dose of 3 grains of ferrous sulphate for four months. A dose of 6 grains daily for two months did not cure the more severe anæmias, but this dose for four months had no advantage over one of three grains. It may reasonably be objected that it would be more economical to give a therapeutic dose of iron for a shorter period, for even at the lower dosage level each man received 336 grains of ferrous sulphate. The advantage of the prolonged small dose is that unlike a therapeutic dose, it produces no untoward symptoms whatever, and this is of considerable importance in mass therapy. In the Army, at least in war time, mass therapy must be used, for it is quite impracticable to estimate the hæmoglobin level of every recruit, and clinical judgment is a most uncertain means of detecting anæmia in the young Indian.

There was little difference in the hæmatinic value of the three diets, but the diet in which meat was replaced by a very generous allowance of tinned milk proved least effective, and the diet giving five times the Army scale of meat proved most effective.

There are two possible explanations for the relatively poor hæmatinic value of the milk diet. Firstly, it has been established that a high calcium diet very definitely impairs iron absorption, and we have shown what a large part iron deficiency played in the anæmia of these recruits. Secondly, Whipple has shown, by experiments on grossly protein-deficient anæmic dogs, that milk compares badly with meat in hæmatinic value. We know very little of these hæmatopoietic factors associated with protein and the vitamin B complex. It is impossible to say whether impaired iron absorption or lack of other hæmopoietic factors was responsible for the poor hæmatinic value of the milk diet in this experiment.

From the point of view of the hæmatologist, the trivial, even doubtful, benefit from a great increase in the meat content of the Army diet is certainly not sufficient to justify any attempt to increase the meat ration in a country with such inadequate resources as India.

A small part of the rise in the recruits' hæmoglobin levels was due to the greater altitude of the Training Centre (1,700 feet above sea-level) compared with their homes, which were in areas less than 600 feet above sea-level. The hæmoglobin increase in men whose initial hæmoglobin level was 16 g. per 100 ml. or over was probably entirely due to this cause. The mean increase in six months, from 16.44 g. to 16.84 g., was in reasonable agreement with Fitzgerald's law which postulates an increase of about 2.8 per cent in the hæmoglobin level for every 1,000 feet increase in altitude.

Hookworm infestation became of absolutely no importance when these recruits were placed on an adequate diet. Chandler (1927), in his classical survey of hookworm infestation in India, said of the races from which these recruits were drawn, 'There is probably more injurious hookworm infestation in South India than in all other parts of India combined'. He also, of course, stressed that even in this part of India only rare and exceptionally heavy infestations will produce anæmia in the absence of other causative factors, of which malnutrition is the most important. In our preliminary survey of these recruits we found that it was only in the more under-nourished minority that infestation was correlated with anæmia.

During six months' observation we have further found that young men even from this most heavily infested part of India are quite unaffected by hookworms when they are given a good diet. Whether they received iron or not, the anæmia of men with even quite heavy infestations improved as quickly as that of men whose infestation was inapparent. It is the custom in the Indian Army to worm all recruits, but in view of our findings it would appear difficult to justify this procedure unless it is postulated that hookworms may produce ill effects other than anæmia.

We found that three diseases, malaria, septic infections of the areolar tissues, and acute tonsillitis, were prominent in their adverse effect on hæmoglobin regeneration. Of these the first two at least must be important in the ætiology of anæmia in rural India—malarial infection is nearly universal, and few recruits cannot show scars to mark the site of a chronic ulcer or whitlow. When men leave their homes to find employment in the Army or coolie gangs, malaria in particular becomes of outstanding importance.

We may conclude by re-emphasizing the benefit to the recruit of reinforcing a good diet with iron therapy. Three grains of ferrous sulphate daily for four months is enough, and an even smaller dose might suffice. The control of anæmia is only one of many reasons for the strict control of malaria in the Army, but we have shown that the seeming importance of hookworm infestation may easily be exaggerated when the diet is adequate.

SUMMARY.

1. An account is given of an experiment on nutritional anæmia in 800 Indian Army recruits.
2. All anæmia improved under the influence of the Army diet, but without iron medication the severer cases of anæmia were still not cured after six months.
3. A daily dose of 3 grains of ferrous sulphate cured all anæmia in four months. A daily dose of 6 grains did not cure the severer grades of anæmia in two months, and when given for four months was not more effective than a daily dose of 3 grains.
4. Three diets were tested, identical in composition with the standard Army ration except in their meat and milk content.
5. A lacto-vegetarian diet giving 54 ounces of milk a day was definitely inferior, judged by the hæmoglobin response, to a diet giving 10 ounces of meat daily.
6. By the same criterion, the diet giving 10 ounces of meat daily was little if at all better than the standard ration diet giving 2 ounces of meat daily.
7. Hookworm infestation did not interfere with hæmoglobin regeneration whether iron was given or not.
8. Malaria, septic infections of the areolar tissues, and acute tonsillitis significantly depressed hæmoglobin regeneration.
9. Recruits who gained in height during the experiment had a relatively poor hæmatological response.

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VITAMIN B₁ (THIAMIN) CONTENT OF INDIAN FOODSTUFFS.

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OVER twenty years ago, McCarrison investigated the vitamin B₁ content of Indian cereals (McCarrison and Norris, 1924; McCarrison, 1928). The method of estimating vitamin B₁ was based on the growth of pigeons and rats on diets in which cereals were the principal ingredients. Owing to the absence of any unit for expressing the vitamin B₁ potency at that time, these investigations did not yield quantitative values for the vitamin content of individual foods. The other investigations on the vitamin B₁ content of Indian foods are those of Ghosh and Guha (1933, 1934), Wilson, Ahmad and Guha (1937), Aykroyd, Krishnan, Passmore and Sundararajan (1940), Passmore and Sundararajan (1941), Swaminathan (1942a), Madhava Rao, Ramachandran and Rao (1942), and Bhagvat (1943).

Ghosh and Guha (*loc. cit.*) and Wilson *et al.* (*loc. cit.*) assayed about 30 to 40 foods using the rat-growth method and compared the potency of test substances with that of the international vitamin B₁ standard. Aykroyd *et al.* (*loc. cit.*), Swaminathan (*loc. cit.*), Madhava Rao *et al.* (*loc. cit.*) and Bhagvat (*loc. cit.*) using modifications of the thiochrome method of Jansen (1936) assayed the thiamin content of 30 to 40 foods. All these investigations have covered only about 50 foods, including many cereals and pulses, a few vegetables, fruits and fish. No data are available for the vitamin B₁ content of many common nuts and oil-seeds, vegetables and fruits. The present work was undertaken to fill this gap. The vitamin B₁ content of about 150 common Indian foods is recorded in this paper. These include the data published already by the author for about 30 foods (Swaminathan, 1942a, 1942b).

METHOD USED.

The method previously published by the author (Swaminathan, 1942a) with some modifications was followed in the present study. The modifications consisted of: (i) enzymic hydrolysis of any phosphorylated vitamin B₁ present in extracts of foods of vegetable origin using takaphosphatase present in takadiastase (although thiamin exists mostly in the free form in foods of vegetable origin, the absence of phosphorylated thiamin in these foods has not yet been definitely established: hence this step was included for all foods), (ii) the use of iso-amyl alcohol instead of iso-butyl alcohol (as iso-butyl alcohol was not available) for preliminary washing of the extracts and for the extraction of the thiochrome, and (iii) the determination of the thiochrome by visual comparison according to the technique of Harris and Wang (1941).

RESULTS.

The results are given in Table I. The values recorded in this paper for Indian foods correspond fairly closely, with those reported by Leong (1940) for Malayan foods, by Harris

TABLE I.
Vitamin B₁ (thiamin) content of foods.

Common English name.	Botanical name.	Vitamin B ₁ (microgram per gramme).
CEREALS AND CEREAL PRODUCTS:		
Barley, whole ...	<i>Hordeum vulgare</i>	4.5
Barley, pearl ...	"	1.0
Italian millet ...	<i>Setaria Italica</i>	5.1

TABLE I—*contd.*

Common English name.		Botanical name.	Vitamin B ₁ (microgram per gramme).
ROOTS AND TUBERS :			
Beet root	...	<i>Beta vulgaris</i>	0·4
Carrot	...	<i>Daucus carota</i>	0·4
Colocasia	...	<i>Colocasia antiquorum</i>	0·9
Onion, big	...	<i>Allium cepa</i>	0·8
Onion, small	...	„	0·7
Parsnip	...	<i>Pastinaca sativa</i>	0·6
Potato	...	<i>Solanum tuberosum</i>	1·2
Radish, white	...	<i>Raphanus sativus</i>	0·6
Radish, pink	...	„	0·5
Sweet potato, pink	...	<i>Ipomoea batatas</i>	0·8
Tapioca, dry	...	<i>Manihot utilissima</i>	0·6
OTHER VEGETABLES :			
Ash gourd	...	<i>Benincasa cerifera</i>	0·4
Bitter gourd	...	<i>Momordica charantia</i>	1·8
Brinjal	...	<i>Solanum melongena</i>	0·5
Broad beans	...	<i>Dolichos lablab</i> var. <i>lignosus</i>	0·8
Cauliflower	...	<i>Brassica oleracea botrytes</i>	1·0
Cow pea, green and tender (with pods).	...	<i>Vigna catieng</i>	1·2
Cucumber	...	<i>Cucumis sativus</i>	0·3
Drumstick	...	<i>Moringa oleifera</i>	0·5
French beans	...	<i>Phaseolus vulgaris</i>	0·8
Indian gooseberry	...	<i>Phyllanthus emblica</i>	0·3
Jack, tender	...	<i>Artocarpus integrifolia</i>	0·5
Knol-khol	...	<i>Brassica oleracea caulorapa</i>	0·5
Ladies finger	...	<i>Hibiscus esculentus</i>	0·6
Mango, green	...	<i>Mangifera indica</i>	0·4
Papaya, raw	...	<i>Carica papaya</i>	0·3
Potola gourd	...	<i>Trichosanthes dioica</i>	0·4
Peas, green	...	<i>Pisum sativum</i>	2·5
Plantain flower	...	<i>Musa paradisiaca</i>	0·5
Plantain, green	...	„	0·4
Plantain stem	...	„	0·2

TABLE I—*contd.*

Common English name.	Botanical name.	Vitamin B ₁ (microgram per gramme).
OTHER VEGETABLES :— <i>contd.</i>		
Pumpkin, yellow (ripe)	... <i>Cucurbita maxima</i>	0·6
Snake gourd	... <i>Trichosanthes anguina</i>	0·4
Sword beans	... <i>Canavalia ensiformis</i>	0·8
Tomato, green	... <i>Lycopersicum esculentus</i>	0·5
Turnip, white	... <i>Brassica rapa</i>	0·4
Water chestnut	... <i>Trapa bispinosa</i>	0·5
FRUITS :		
Apple	... <i>Pyrus malus</i>	0·4
Banana	... <i>Musa sapientum</i>	0·4
Dates, preserved	... <i>Phoenix dactylifera</i>	0·8
Grapes	... <i>Vitis vinifera</i>	0·4
Grape fruit	... <i>Citrus grandis</i> var. <i>maxima</i>	0·3
Guava	... <i>Psidium guajava</i>	0·3
Jack fruit	... <i>Artocarpus integrifolia</i>	0·3
Lemon juice	... <i>Citrus medica</i> var. <i>limonum</i>	0·2
Lime juice	... <i>Citrus medica</i> var. <i>acid</i>	0·2
Mango, ripe	... <i>Mangifera indica</i>	0·4
Orange juice	... <i>Citrus aurantium</i>	0·5
Papaya	... <i>Carica papaya</i>	0·4
Peaches, white	... <i>Amygdalis persica</i>	0·2
Pears, country	... <i>Pyrus communis</i>	0·2
Pears, English	... <i>Pyrus achras</i>	0·2
Plantain, ripe	... <i>Musa paradisiaca</i>	0·5
Plums	... <i>Prunus domestica</i>	0·3
Pomelo	... <i>Citrus decumana</i>	0·3
Raisins (preserved)	... <i>Vitis vinifera</i>	0·6
Strawberry	... <i>Fragaria grandiflora</i>	0·3
Tomato, ripe	... <i>Lycopersicum esculentum</i>	0·5
Tamarind, raw, fresh	... <i>Tamarindus indicus</i>	0·2
Tamarind (preserved)	...	0·6
Water melon	... <i>Citrullus vulgaris</i>	0·2

TABLE I—concl'd.

Common English name.	Botanical name.	Vitamin B ₁ (microgram per gramme).
MILK AND EGGS :		
Milk, cow's		0.4
Milk, buffalo's		0.4
Milk, evaporated		0.8
Milk, sweetened condensed		0.6
Whole-milk powder		2.5
Skimmed milk powder		3.2
Eggs, whole, hens'		1.3
Eggs, whole, ducks'		1.2
FLESH FOOD :		
Beef (muscle)		1.4
Fish, specimen 1		0.8
Fish, specimen 2		1.0
Fish, specimen 3	(<i>Catla catla</i>)	0.8
Fish, specimen 4	(<i>Labeo rohita</i>)	0.9
Fish, specimen 5	(<i>Notopterus chitala</i>)	1.2
Liver, sheep		3.6
Mutton (muscle)		1.6
Pork (muscle)		7.8
MISCELLANEOUS :		
Betel leaf		0.7
Sago		0.1
Jaggery (palmyra)		0.2
Jaggery (sugarcane)		0.2
Yeast, dried, baker's		22.5
Yeast, dried, brewer's 1		42.1
Yeast, dried, brewer's 2		45.8
Yeast dried, brewer's 3		36.4
Yeast dried, brewer's 4		33.5
'Food' yeast, dried (grown in <i>Torula utilis</i> molasses-salts medium) :		
Sample 1		33.5
Sample 2		28.4
Sample 3		32.5
Yeast, dried, distillery, sample 1		10.4
Yeast, dried, distillery, sample 2		10.8
'Marmite', an yeast extract		13.0
'Vegamite', an yeast extract		38.0

and Wang (*loc. cit.*) for English foods. by Munseil (1943) and Lane, Johnson and Williams (1942) for American foods.

The results show that vitamin B₁ is fairly widely distributed in many natural products. A summary of the results is given below :—

Cereals.—Whole cereals rank first, as all authorities agree, as the most important source of vitamin B₁ in human diets. Whole wheat, unmilled rice, whole barley, oats, and the common millets, e.g. cambu (*Pennisetum typhodeum* Rich.), cholam (*Sorghum vulgare pers.*), ragi (*Eleusine coracana* Gaertn.) and Italian millet, contain from 4 to 5 micrograms of thiamin per gramme. The vitamin B₁ content of milled cereals, e.g. milled rice, or white flour, depends upon the degree of milling to which these products have been subjected and also whether the rice is parboiled or not. A point of great practical importance is that parboiled milled rice is a good source (2 to 2.5 micrograms per gramme) in contrast to raw milled rice which is a poor source (0.8 to 1 microgram per gramme). These results are consistent with the fact that beri-beri as a public health problem occurs only among poor people consuming raw milled rice (Aykroyd *et al.*, *loc. cit.*; Swaminathan, 1942c). Certain other highly milled cereals, e.g. refined wheat flour (white flour), pearl barley, and refined corn flour, are also poor sources of this vitamin. In this connection it is of interest to record that Aykroyd (1930) has reported that beri-beri occurred among fishermen in Newfoundland and Labrador, consuming a diet largely composed of refined wheat flour.

Sixteen ounces of a whole cereal will supply about 1,500 to 2,200 micrograms of thiamin, whereas a similar quantity of a milled cereal will provide only 400 to 500 micrograms.

Pulses.—Dried pulses are good sources of thiamin, containing about 4 to 7 micrograms per gramme. A daily intake of 3 to 4 ounces of pulses will provide about 400 to 700 micrograms of thiamin.

Nuts and oil-seeds.—Nuts and oil-seeds are in general good sources of thiamin. Gingelly seeds and pea-nuts are the richest, containing 10 and 8 micrograms per gramme. Pistachio, walnut and cashew nut are also good sources containing about 6 to 7 micrograms per gramme. Fresh coco-nut is a poor source (1.1 microgram per gramme). Two ounces of gingelly seeds or ground-nut will supply about 400 to 500 micrograms of thiamin.

Vegetables.—Vegetables in general (with the exception of green peas, and other green legumes) are poor sources of thiamin containing from 0.1 to 1 microgram per gramme. Green peas are good sources (3 micrograms per gramme). Green leafy vegetables, roots and tubers are in general better sources than other vegetables, e.g. gourds, etc. In general on account of their high water content and great bulk vegetables are not important dietary sources of thiamin.

Fruits.—Fruits are poor sources of thiamin, containing from 0.2 to 0.8 microgram per gramme. Four ounces of fruits will supply only 30 to 80 micrograms of thiamin.

Milk and eggs.—Fresh cow's and buffalo's milk are fair sources, containing about 0.4 to 0.5 micrograms per gramme. An intake of 16 ounces of milk will supply about 200 to 250 micrograms. Milk is an important source of thiamin in the diet of children. Eggs are also fair sources.

Flesh foods.—Among flesh foods, lean pork is the richest source containing about 7 micrograms per gramme. Liver ranks next in order of merit, with a value of 4 to 5 micrograms. Meat and fish are fair sources. Four ounces of lean pork will supply about 700 to 800 micrograms of thiamin, while a similar quantity of meat or fish will provide only 100 to 120 micrograms.

Dried yeast.—Dried yeast, though not a common article of food is a valuable supplement to the human dietary as a source of vitamin B complex and protein. Hence it was thought of interest to assay yeasts prepared under different conditions. Dried brewer's yeast and food yeast (*Torula utilis* grown in molasses-salts medium) are good sources containing from 25 to 40 micrograms per gramme. Daily intake of 1 ounce of dried food yeast will supply about 700 to 1,200 micrograms. Distillery yeast, a by-product in the manufacture of rectified spirit, is only a fair source containing 10 to 12 micrograms per gramme.

Vitamin B₁ requirements and important food sources of vitamin B₁.

According to the Food and Nutrition Board of the National Research Council, U.S.A., the human requirements of vitamin B₁ are as follows: For adult males 1.5 mg. to 2.3 mg.; for adult females 1.2 mg. to 1.8 mg.; for pregnant and nursing women 1.8 mg. and 2.3 mg.; for children, ranging from 0.4 mg. for those under one year to 2.3 mg. for boys between 14 and 18. It will be of interest to know the important sources of thiamin in a well-balanced diet for adults. Table II shows the composition of a well-balanced diet (Indian) for adults and the quantity of thiamin derived from each group of foodstuffs. It is evident from the table that *whole cereals and pulses* are the most important source of vitamin B₁ in the Indian diets.

TABLE II.

The contribution of vitamin B₁ by different groups of foodstuffs to a well-balanced diet for adults.

Name of foodstuff.	Quantity (ounces) per day.	Vitamin B ₁ (microgram).
<i>Whole cereals :</i>		
Unmilled rice, whole wheat, or millets	16	1,200 to 1,800
<i>Pulses :</i>		
Bengal gram, green gram, or red gram or massur dhal.	3½	400 to 700
<i>Vegetables :</i>		
Green leafy	4	40 to 60
Non-leafy	4	50 to 100
<i>Fruits :</i>		
Tomato, orange, etc.	4	40 to 60
<i>Milk :</i>		
Cow's, whole	16	200 to 250
Meat	4	100 to 150
or		(Pork 700 to 800)
Fish	4	80 to 100
or		
Egg (one)	1½	60 to 80

SUMMARY.

1. The vitamin B₁ content of about 150 common Indian foods has been determined by the thiochrome method.

2. Of the foods tested, dried brewer's yeast, rice polishings (from raw paddy) and wheat germ are rich sources of thiamin.

3. Among the cereals, whole cereals, e.g. whole wheat, millets, whole barley, unmilled rice and oats, are good sources containing 3 to 4 times as much thiamin as milled cereals, e.g. white flour, pearl barley and raw milled rice. Parboiled milled rice contains two to three times as much thiamin as that present in raw milled rice.

4. All dried pulses are good sources of thiamin. The common nuts and oil-seeds (with the exception of coco-nut) are also rich sources.

5. Cow's and buffalo's milk, eggs and flesh foods are fair sources; lean pork is a rich source.

6. Fresh vegetables (with the exception of green peas) and fruits are poor sources of thiamin.

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TOXIC EFFECTS IN GUINEA-PIGS OF DIETS CONTAINING LARGE PROPORTIONS OF *LATHYRUS SATIVUS*.

BY

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THE disease known as lathyrism has long been ascribed to the consumption of the seeds of *Lathyrus sativus* or 'khesari' in large quantities for several months. Outbreaks of lathyrism have occurred in India from time to time; a good review on the subject is found in a recent paper by Shourie (1945), who carried out, on behalf of these Laboratories, an investigation on lathyrism during the 1944-45 outbreak of this disease in Bhopal State. The results reported by Shourie (*loc. cit.*) point to the existence of a toxin in lathyrus seeds, which was suggested to be responsible for producing lathyrism in man. The mode of action and the nature of the toxin are not known. It was felt to be of interest to study this problem with guinea-pigs as test animals. It was proposed to put them on diets containing large proportions of khesari gram, obtained from those villages in Bhopal State and the Central Provinces where cases of lathyrism had occurred, with a view to attempt to induce in them a condition resembling human lathyrism. Some years ago, feeding tests with pure stocks of khesari and that of 'acta' (*Vicia sativa*) had been carried out in these Laboratories with rats as test animals (McCarrison, 1927; McCarrison and Krishnan, 1934). No deleterious effects could be demonstrated in these species of animals as a result of feeding them on diets consisting largely of these two seeds. Contrary to these findings, Geiger, Steenbock and Parsons (1933) and Lewis and Esterer (1943) reported to have produced lathyrism in rats. The species of lathyrus seeds used by these authors, however, was the *Lathyrus odoratus* and not *Lathyrus sativus*. Certain Spanish workers (Jamenez, Vivanco and Castro, 1943; Aldama-Truchuelo and Mateo, 1944) have reported production of a neurological syndrome in rats (resembling lathyrism in man) fed solely on chick peas (*Lathyrus sativus*). While, Anderson, Howard and Simensen (1925) observed no deleterious effect in monkeys and ducks resulting from the use of khesari as food for these animals; acta, however, produced in monkeys symptoms similar to those observed in cases of human lathyrism. These conflicting findings are hard to explain.

EXPERIMENTAL.

The seeds of *Lathyrus sativus* or khesari were obtained from Bhopal State and Central Provinces through the kindness of the Medical Officer, Bhopal State, and the Director of Public Health, Central Provinces and Berar, respectively. The samples which were reported to be free from *Vicia sativa* L. (acta) were collected from villages, in which cases of lathyrism had occurred.

First experiment.—Male guinea-pigs weighing from 450 g. to 500 g. were used as experimental animals. They were kept on the laboratory stock diet rich in all the vitamins for a week and then put on the experimental diets. Two such diets were used, one consisting of coarsely powdered dry Bengal gram and the other coarsely powdered khesari gram. The animals receiving the Bengal gram diet served as the control, since this diet has been shown (Bhagvat and Rao, 1942) to be adequate for the promotion and maintenance of growth of guinea-pigs. The composition of these diets is given in Table I.

The diets were moistened with water and given in the form of balls. Water was provided for drinking. Each animal was given a daily supplement of 5 mg. of ascorbic acid. The experiment was repeated in two separate batches, each batch consisting of 20 guinea-pigs. They were divided into two groups, one receiving the Bengal gram diet and the other the khesari gram diet.

TABLE I.
Composition of the experimental diets.

Components.				DIETS.	
				Bengal gram parts.	Khesari gram parts.
Bengal gram	80.0	...
Khesari gram	80.0
Crushed oats	8.0	8.0
Skimmed milk powder	5.0	5.0
Dried brewer's yeast	1.0	1.0
NaCl	1.0	1.0
Gingelly oil	4.0	4.0
Shark-liver oil (containing 1,000-1,500 I.U./g.)				1.0	1.0
				100.0	100.0

Each animal was confined in a separate cage under conditions of scrupulous cleanliness and was weighed weekly. The average body-weights of 10 animals in each group are shown diagrammatically in Fig. 1 :—

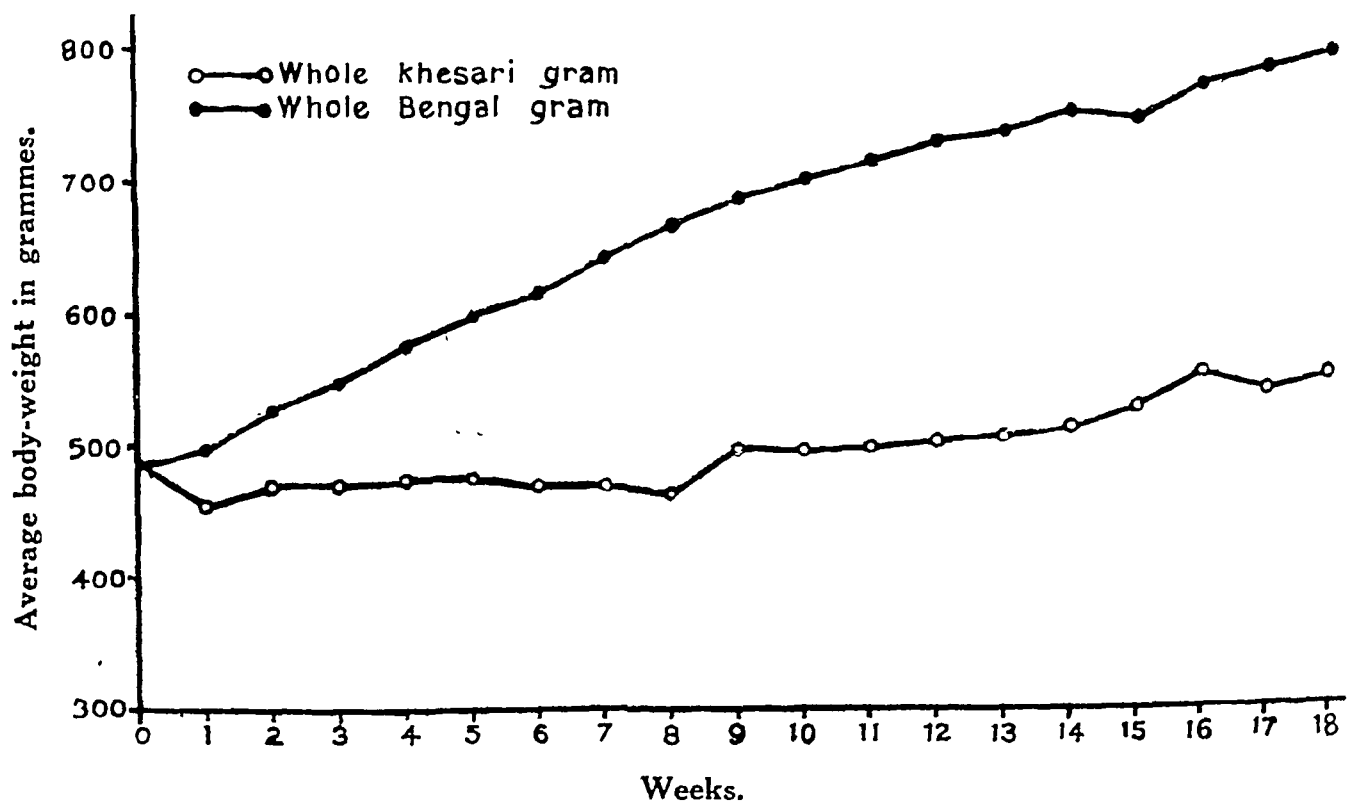


Fig. 1.—Average weight curves of guinea-pigs receiving diets containing 80 per cent Bengal gram and 80 per cent khesari gram respectively.

The animals receiving the Bengal gram diet grew steadily and remained in perfect health; while those receiving the khesari gram diet did not show any significant increase in weight. They developed alopecia and a slight dermatitis within the first few weeks of the experiment, and their coats were dirty. The animals showed an extreme reluctance to move about. In no experiment, however, did they develop paralysis of the hind legs, but they remained in this condition throughout the experimental period. Some 50 per cent of the animals on khesari gram died suddenly and the remaining ones and those receiving the Bengal gram diet were sacrificed simultaneously and were subjected to a post-mortem examination. A naked-eye examination of the tissues from animals on khesari gram diet on post mortem revealed marked hæmorrhages in the adrenals in certain cases and the livers and the kidney were not normal. (They were slightly enlarged, soft in consistency and pale in colour. On sectioning, the cortex and the medulla in the kidney could not be differentiated.) The tissues and the nerves, however, were not subjected to a thorough histological examination. The results of this experiment in general appear to suggest that there is a factor toxic to guinea-pigs, in the lathyrus seeds, the ill-effects of which could not be prevented by the inclusion in the diet of liberal amounts of the known vitamins.

Second experiment.—This experiment was a repetition of the first experiment with the difference that instead of male guinea-pigs, younger guinea-pigs—male and female—weighing 200 g. to 250 g. were used, other experimental conditions remaining the same. The experiment was of interest, since, in humans, the adult man is found to be more susceptible to lathyrism than either a woman or a child. The harmful effects of the khesari gram when it formed a part of the diet, were observed in both male and female guinea-pigs and the symptoms manifested themselves in approximately the same time as in the animals in the first experiment. These findings appear to suggest that the age and the sex of the animal do not seem to influence its susceptibility to deleterious effects of khesari gram.

Third experiment.—This experiment was planned with a view to study the effect of (1) the removal of the outer husk of the khesari gram, since a theory has been put forward that the only harmful part of this grain is the husk (private communication, Director of Public Health, Central Provinces and Berar) and (2) the substitution of Bengal gram in the diet by other grams, viz. the green and the black grams, on the growth of guinea-pigs. The composition of the diet otherwise was the same as described in Table I. Guinea-pigs distributed equally between the two sexes weighing from 300 g. to 350 g. were used. The experiment was repeated in two separate batches, each batch consisting of 16 animals. They were grouped as follows:—

- 1ST GROUP: ... 8 guinea-pigs (4 in each batch) receiving the whole khesari gram diet.
- 2ND GROUP: ... 8 guinea-pigs (4 in each batch) receiving the husked khesari gram diet.
- 3RD GROUP: ... 8 guinea-pigs (4 in each batch) receiving the green gram diet.
- 4TH GROUP: ... 8 guinea-pigs (4 in each batch) receiving the black gram diet.

Each guinea-pig was given 5 mg. of ascorbic acid daily.

The results of this experiment are shown in Fig. 2 (p. 302).

The growth of guinea-pigs on either green gram or black gram diet was comparable with that obtained when the animals were fed the Bengal gram diet, and these animals remained in good health throughout the experimental period. These results show that both the green gram and the black gram diets were adequate for the growth of this species of animals. The diets containing khesari gram (with and without husk) did not promote growth. All the animals developed the same symptoms as described in the first experiment. Thus, the removal of the husk does not appear to remove the harmful principle of the gram. Eight out of 16 guinea-pigs died in the course of this experiment; amongst those died, 6 developed deep trophic ulcers in the hind legs in about 10 to 16 weeks on the diet containing khesari gram. These ulcers could not be cured by intramuscular injections of thiamin or by inclusion of liberal

amounts of all the B vitamins in the diet. Whether these ulcers were due to the degeneration or impairment of the sensory nerves is not known. Only a thorough histopathological examination of the sensory and the motor nerves and of the spinal cord will settle this point.

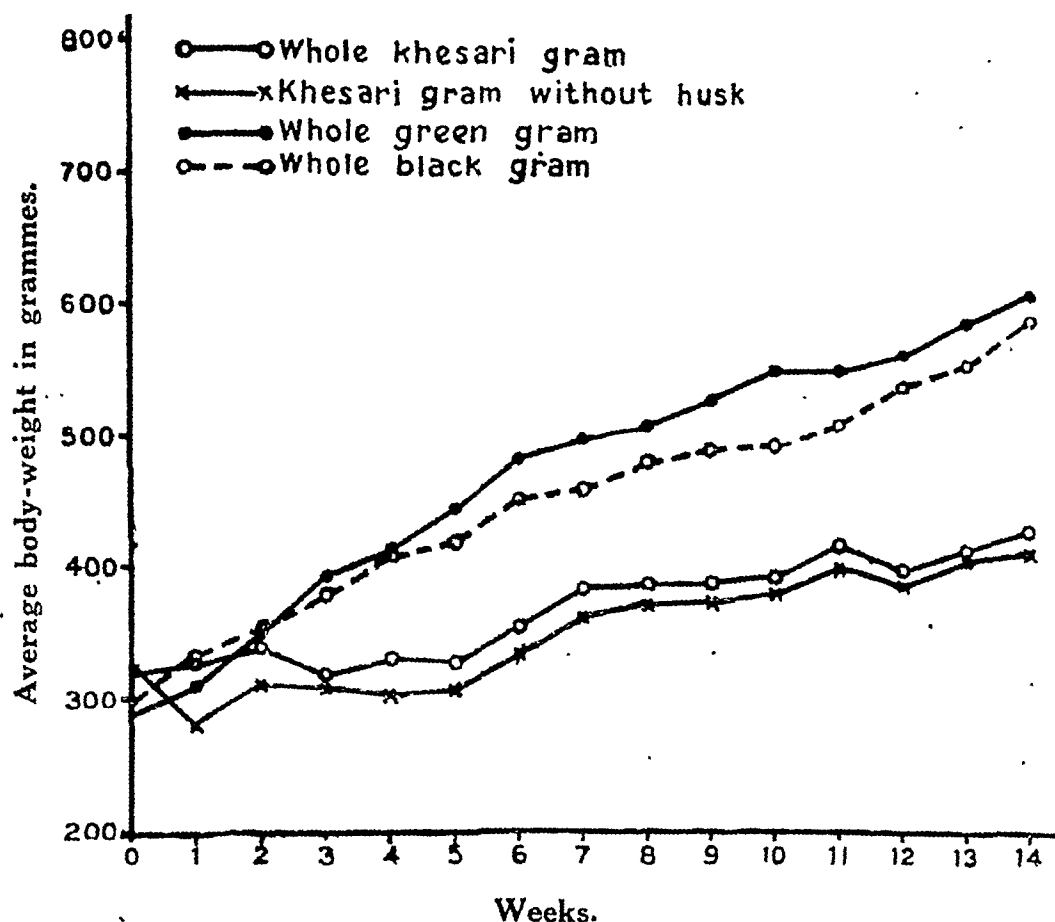


Fig. 2.—Effect of inclusion of green gram, black gram and khesari gram (with and without husk) in the diet on the growth of guinea-pigs.

Fourth experiment.—In this experiment an attempt was made to find out what proportion of khesari gram could be included in the diet of guinea-pigs to lead to the danger of the disease. The composition of the different diets used is given in Table II. (Atta was used in this experiment, since, in places, where khesari gram is consumed, it is often mixed with wheat.)

TABLE II.
Composition of the experimental diets.

Components.	D I E T S.			
	P a r t s.			
	1	2	3	4
Khesari gram	75.0	50.0	30.0
Atta ...	80.0	5.0	30.0	50.0
Skimmed milk powder ...	5.0	5.0	5.0	5.0
Crushed oats ...	8.0	8.0	8.0	8.0
Gingelly oil ...	4.0	4.0	4.0	4.0
Dried brewer's yeast ...	1.0	1.0	1.0	1.0
NaCl ...	1.0	1.0	1.0	1.0
Shark-liver oil ...	1.0	1.0	1.0	1.0
	100.0	100.0	100.0	100.0

Ascorbic acid 5 mg. per animal per day.

Twenty-four guinea-pigs, weighing from 400 g. to 450 g., distributed equally between the two sexes, were used in this experiment. They were divided into 4 groups (6 animals in each group). The 1st, 2nd, 3rd and 4th groups were given Nos. 1, 2, 3 and 4 diets respectively. The average body-weights of the animals in the different groups are shown in Fig. 3 :—

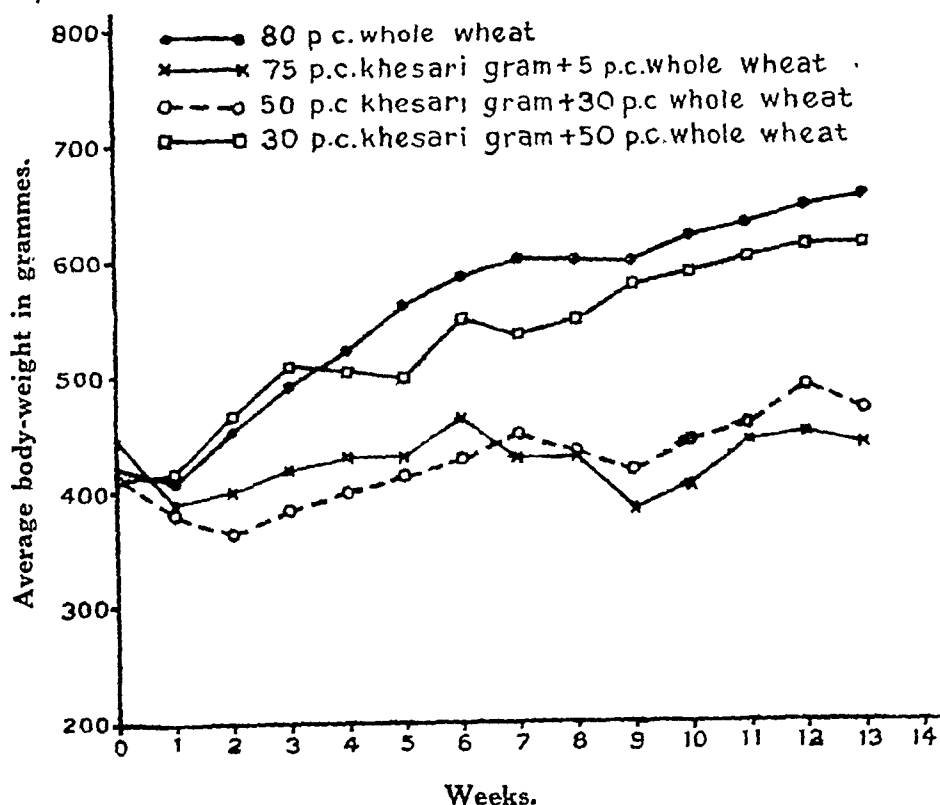


Fig. 3.—Average weight curves of guinea-pigs receiving diets containing varying proportions of wheat and khesari gram.

Of the four diets tested, diets Nos. 1 and 4 appear to be adequate for the growth of guinea-pigs. Diets Nos. 2 and 3 were injurious to these animals; inclusion of wheat at a level of 30 per cent in diet No. 3 does not appear to protect them against the harmful effects of khesari gram. In groups 2nd and 3rd, 6 animals out of 12 died in 8 to 12 weeks from the start of the experiment; of those died, 4 developed deep trophic ulcers in the hind legs. This experiment shows that inclusion of khesari gram in the diet at a level of 30 per cent does not produce toxic effects in guinea-pigs. These observations fall in line with the suggestions of Buchanan (1904) that khesari gram at 30 per cent level in the diet was not harmful to man.

DISCUSSION.

The experiments reported in this paper show that the guinea-pigs grew properly on diets consisting largely (80 per cent level) of Bengal gram, green gram and black gram. When, however, khesari gram was included in the diet at 80, 75 and 50 per cent levels, a morbid condition was produced in these animals. They did not grow properly, developed alopecia, a slight dermatitis, and in some cases acute trophic ulcers in the hind legs. No obvious paralysis of the hind legs was, however, observed. On death, the tissues from these animals were subjected to post-mortem examination, which revealed certain changes in the liver, the kidney and the adrenals. These changes were identical in every case. The symptoms described above, however, did not occur in animals fed a mixed diet containing 30 per cent khesari gram and 50 per cent wheat.

McCombie Young (1927) has suggested 'that lathyrism may be to some extent a deficiency disease which is produced in persons living in a state of nutritional instability on a diet noticeably lacking in vitamin A; by prolonged ingestion of a legume, the amino acids of whose proteins are unsuitable as a diet and perhaps specially harmful, which is itself deficient in fat-soluble A'. Mellanby (1934) has suggested that lathyrism is due to the presence of a neurotoxin in khesari gram; the deleterious effects of which were enhanced in vitamin A deficiency and prevented by inclusion of vitamin A or carotene in the diet. The possibility of such a deficiency occurring in the experimental diets used in this investigation has to be ruled out, since all the diets contained adequate amounts of all the known vitamins. Thus, the morbid condition induced in guinea-pigs by feeding them diets containing a large proportion of khesari gram may be attributed to the presence of a toxin in these seeds. As regards the nature and mode of action of this toxin, nothing is known at the moment and is a matter for future investigation.

The question whether the morbid condition induced in guinea-pigs is related to lathyrism in man must be left undecided until further investigations and a thorough study of the pathology of the two diseases proves or disproves their identity.

SUMMARY.

1. The effect of feeding diets consisting largely of Bengal gram, green gram, black gram and khesari gram and containing adequate quantities of the known vitamins on the growth of guinea-pigs has been investigated.

2. Guinea-pigs grew normally on the diets containing Bengal, green and black, grams. They, however, did not grow when khesari gram (with or without husk) was included in the diet at 80, 75 and 50 per cent levels; the animals were extremely reluctant to move about; they developed alopecia, a slight dermatitis and in certain cases deep trophic ulcers in the hind legs. No obvious paralysis of the hind legs was observed. These symptoms were identical in every case.

3. Guinea-pigs on a mixed diet consisting of 30 per cent khesari gram and 50 per cent wheat did not develop these symptoms, and remained in good health.

4. Young and adult and male and female guinea-pigs were equally affected.

5. The harmful effects observed with khesari gram could not be alleviated by the addition of vitamins (vitamins A, C and those of the B group) to the diet and thus they are attributed to the presence of a toxin in these seeds.

Thanks are due to Mr. M. Susai, Laboratory Assistant, for the technical assistance rendered in this investigation.

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STUDIES ON SOME DEXTROROTATORY HYDROCUPREIDINE DERIVATIVES.

Part IV.

COMPARATIVE SPERMICIDAL EFFECTS ON CAVY (GUINEA-PIG) SPERMS.

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INTRODUCTION.

At the instance of late Colonel H. W. Acton and Colonel Sir Ram Nath Chopra of the School of Tropical Medicine, Calcutta, a study of the dextrorotatory hydrocupreidine derivatives was undertaken a few years ago by one of the authors (B. M.) in collaboration with co-workers. Investigations concerning hæmolytic activity (Chopra, Mukerji and Chakravarty, 1938), comparative action on digestive enzymes (Mukerji and Iyengar, 1938) and comparative lethal effects on *Paramœcium caudatum* (Mukerji, Dutta and Ganguly, 1942) of hydrocupreidine and its higher homologues have already been reported. From the earlier studies on their hæmolytic effects in general and the inhibitory effects on digestive enzymes of the higher members of the series in particular, it became apparent that these derivatives might not prove suitable for systemic medication, either by the oral or the parenteral routes. A general study of their pharmacological properties with particular reference to the circulatory and the respiratory systems (unpublished) also failed to reveal any desirable features in their actions (using the dosage range in which these salts could be safely administered), which might indicate therapeutic promise in the above directions.

The rather powerful effects in high dilutions of these derivatives on paramœcia, however, opened up the possibility of their being used as protozoocidal, spermicidal and perhaps bactericidal remedies on surface application. Work was, therefore, concentrated in these directions. In the present paper, an attempt is made to report the findings in connection with the spermicidal potency of some hydrocupreidine derivatives.

Baker (1931a, 1931b), Baker and Ranson (1934), and Baker, Ranson and Tynen (1937) have investigated the spermicidal values of a large number of pure chemicals and commercial contraceptives available either as suppositories or jellies. Most of the chemicals tested by him proved to be insufficiently soluble, unstable in spermicidal concentrations or liable to stain the tissues or fabrics used in feminine hygiene. It was further observed that the majority of commercial suppositories or jellies were not sufficiently strongly spermicidal to enable users to rely on them implicitly. There is room, therefore, for the elaboration of newer spermicides of high and immediate efficacy. Some members of the hydrocupreidine series, as will be seen from the results recorded herein, appear to offer promise of being utilized as satisfactory spermicides, either alone or in combination with other pharmaceutical ingredients in suitable vehicles.

EXPERIMENTAL METHODS.

The investigation was conducted with guinea-pig sperms, firstly because these are readily available and secondly because it is stated on good authority (Baker, 1932b) that these sperms, when suspended in albumin saline, react to spermicides in much the same way as

human spermatozoa. The technique applied was essentially the same as that of Baker, Ranson and Tynen (1937) with slight modification to suit local conditions. Adult male guinea-pigs, not less than 600 grammes in weight, were killed by a blow on the head and the testes dissected out. The tails of both the epididymes were cut and the contents squeezed out into a suitable solution (albumin saline). The sperms were thoroughly mixed with the diluting fluid. The subsequent procedure was carried out at a constant temperature of $37^{\circ}\text{C.} \pm 1^{\circ}\text{C.}$ The stock solution of the substance to be tested was then diluted to required concentration in normal saline and 0.3 c.c. of this solution at 37°C. was mixed with 0.3 c.c. of the sperm suspension at the same temperature. The substance is now half the concentration at which this was first made up. The sperms were examined on the warm stage ($37^{\circ}\text{C.} \pm 1^{\circ}\text{C.}$) of the microscope at the end of 5 minutes and 30 minutes, and their activities recorded as follows:—

‘3’ indicates that the majority of the sperms are moderately or very active.

‘2’ indicates that 10 per cent of the sperms are moderately active or that the majority are feebly active, or that there is any greater amount of activity that is less than ‘3’.

‘1’ indicates any activity less than ‘2’, including the slightest movement in a single sperm.

‘0’ indicates that examination of 10 microscopical fields fails to reveal the slightest movement in a single sperm.

If the sperms were rendered immobile in 30 minutes, the suspension was diluted three times with ‘alkaline diluting’ fluid* to find out whether they were merely immobilized or really dead. Spermicidal value of a substance was arbitrarily taken as the minimum concentration of that substance which produced death of the sperms in 30 minutes.

Suspension of sperms in albumin saline.—The ‘albumin saline’ for suspension of sperms was made up in the same way as recommended by Baker *et al.* (1934). It was found desirable, however, to change the proportion of egg albumin to sodium chloride (2.3 c.c. egg albumin to 0.7 c.c. of 0.9 per cent NaCl) slightly so as to obtain a thinner suspending fluid (2 parts of egg albumin to 1 part of 0.9 per cent NaCl) allowing of better motility of the sperms.

Preparation of test solutions.—Hydrocupreidine derivatives are either insoluble or sparingly soluble in water. Their corresponding hydrochlorides being more soluble in water, these were used in the experiments. In all cases, the bases were converted into their corresponding hydrochlorides by the addition of calculated amounts of N/1 hydrochloric acid. A 1 per cent (100 mg. 10 c.c.) stock solution was first made and subsequent dilutions were made from this. Some of the higher homologues, e.g. iso-heptyl salt, required more than the calculated amount of hydrochloric acid to dissolve, resulting in a solution with a high acid pH. Parallel studies under optimum pH could not be carried out with these salts even after buffering. These were therefore not used. Ethyl-hydrocupreidine was found to have partially disintegrated and hence discarded from the present study.

Hydrogen-ion concentration of test solution.—The most favourable hydrogen-ion concentration for the activity of sperms was found to be between 7.5 and 9.5. The motility was arrested below pH 6.5 or above 10. The pH of the resulting solutions formed by mixing equal quantities of test solutions and sperm suspended solution lie between 7.6 and 7.8 and this is quite favourable for the activity of sperms. In killing concentrations, none of the salts except hydrocupreidine hydrochloride showed any tendency to precipitate out when mixed with an equal quantity of sperm suspended solution. In a concentration of 1 in 600, i.e. killing concentration, this salt was often unstable and experiments had always to be done with freshly prepared solutions.

* Alkaline diluting fluid is prepared by mixing equal volumes of solutions A and B. Sol. A: $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 6 per cent in water; Sol. B: glucose—3.0 g., $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ —0.6 g., NaCl—0.2 g., KH_2PO_4 —0.01 g., dist. water—100 c.c.

RESULTS.

The spermicidal values of different hydrocupreidine derivatives in relation to that of quinine hydrochloride, which is taken as the standard for comparison, is shown in Table I. In Table II, the corresponding values of the available hydrocupreine derivatives (lævorotatory salts) are given. It will be seen that hydrocupreidine hydrochloride is a weak spermicide having almost the same effect as that of quinine hydrochloride. The addition of an alkyl radicle to the side chain considerably increases the killing concentration of the salt and this increases as we ascend higher up in the series. In this respect, the effect is similar to, and almost parallel with, the hæmolytic and paramœcidal activities of these derivatives reported earlier. The sperms are, however, more resistant to the action of these salts than paramœcium. Normal hexyl hydrocupreidine hydrochloride is somewhat peculiar in its behaviour. This salt, contrary to its position in the homologous series, is found to possess the highest spermicidal value. Compared to quinine hydrochloride, it is approximately one hundred and eighty times stronger (killing concentration is $1/360$ per cent, i.e. 1 in 36,000). No definite relationship, as was observed in the lethality of *P. caudatum*, could be demonstrated between the normal hydrocupreidines and their corresponding isomers. Normal butyl hydrocupreidine hydrochloride is more powerful in its action than its iso-derivative whereas normal amyl hydrocupreidine hydrochloride is less toxic to spermatozoa than its iso-salt. No significant difference existed between dextrorotatory hydrocupreidine derivatives and their corresponding lævorotatory hydrocupreine isomers in their action on guinea-pig sperms.

DISCUSSION.

Hydrocupreidine derivatives, as will be seen from the results obtained, possess remarkable spermicidal power. Baker (1931a, 1931b) investigated a large number of chemicals for their comparative spermicidal values. Substances such as quinine, chinisol, quinine urea hydrochloride and lactic acid, which form the basic substances for the chemical contraceptives in common use, were all found by him to be poor spermicides. The spermicidal values of quinine hydrochloride is $\frac{1}{3}$ per cent; that of chinisol and quinine urea hydrochloride is $\frac{1}{4}$ per cent (1 in 400). Tables I and II show that under identical experimental conditions most of the salts under investigation are much more potent than any of the chemicals referred to above. Methyl hydrocupreidine hydrochloride is about twenty-five times stronger than quinine hydrochloride and N-hexyl hydrocupreidine is about one hundred and eighty times more powerful. The spermicidal power of the latter salt is even higher than mercuric chloride, which, besides quinones (tolu-, butyl-, methoxy-, and parabenzoquinones), was considered by Baker (1932a) to be the strongest spermicide known (killing concentration of mercuric chloride is $1/256$ per cent).

Mercuric chloride, quinones and other similar substances possessing high spermicidal values have little practical utility; either they are injurious to the tissues or stain the tissues when applied or are unstable in effective concentrations. The higher members of the hydrocupreidine series, in effective concentrations, are soluble in water and are not precipitated in the faintly alkaline medium which may be expected to exist inside the vagina. In such concentrations they appear to possess another advantage in that they do not produce any undue local irritant effect on application to the mucous surfaces, such as that of vagina and conjunctiva of the rabbit. Some of these salts, therefore, deserve further trial as constituents for chemical contraceptives.

What really contributes to the high spermicidal power of these salts is not known. But it is interesting to note that the spermicidal values increase up to the hexyl salt with the addition of the carbon and hydrogen atoms to the side chain. Probably the ability of a salt to ionize quickly influences to a certain extent the degree of its activity.

SUMMARY.

1. Spermicidal values of hydrocupreidine derivatives and their corresponding available lævorotatory isomers have been studied on cavy (guinea-pig) sperms and compared with that of quinine hydrochloride.

2. The hydrocupreidine derivatives are powerful spermicides and this action generally increases (up to the hexyl derivative) with the addition of alkyl groups in the side chain.

3. There is no significant difference in the behaviour of dextrorotatory hydrocupreidine derivatives and their corresponding levorotatory isomers, nor is there any definite relationship between the iso- and normal salts.

4. Some of the hydrocupreidine derivatives are very potent spermicides and in view of the high dilutions in which these can exert their action and the absence of local irritant effect on the vaginal mucous membrane, these salts offer promise of being utilized in contraceptive preparations, provided suitable pharmaceutical formulæ in which this action is best elicited could be elaborated. Work is in progress in this direction.

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TABLE I.

Effect of dextrorotatory hydrocupreidine derivatives on cavy (guinea-pig) sperms.

Substance.	Strength of solution.	RESULTS.		pH of test solution.	pH of control solution.	Spermicidal value, per cent.	Potency in terms of quinine hydrochloride which is taken as 1 unit.
		Control solution.	Test solution.				
		5 m. 30 m.	5 m. 30 m.				
Quinine hydrochloride	... { 1 in 200 1 in 400 (1/4)	3 3	2 2	7.8	7.8	{	1.00
Hydrocupreidine hydrochloride	... { 1 in 600 1 in 800 (1/4)	3 3	2 2	7.8	7.8	{	3.00
Methyl hydrocupreidine hydrochloride	... { 1 in 5,000 1 in 6,000 (1/50)	3 3	2 2	7.6	7.8	{	25.00
N-propyl hydrocupreidine hydrochloride	... { 1 in 9,500 1 in 10,000 (1/95)	3 3	2 2	7.7	7.8	{	47.50
Iso-propyl hydrocupreidine hydrochloride	... { 1 in 8,500 1 in 9,000 (1/85)	3 3	2 2	7.7	7.8	{	42.50
N-butyl hydrocupreidine hydrochloride	... { 1 in 19,000 1 in 20,000 (1/190)	3 3	2 2	7.7	7.8	{	95.00
Iso-butyl hydrocupreidine hydrochloride	... { 1 in 17,000 1 in 18,000 (1/170)	3 3	2 2	7.6	7.8	{	85.00
N-amyl hydrocupreidine hydrochloride	... { 1 in 19,000 1 in 20,000 (1/190)	3 3	2 2	7.7	7.8	{	95.00
Iso-amyl hydrocupreidine hydrochloride	... { 1 in 26,000 1 in 27,000 (1/260)	3 3	2 2	7.6	7.8	{	130.00
N-hexyl hydrocupreidine hydrochloride	... { 1 in 36,000 1 in 37,000 (1/360)	3 3	2 2	7.6	7.8	{	180.00
N-heptyl hydrocupreidine hydrochloride	... { 1 in 27,000 1 in 28,000 (1/270)	3 3	2 2	7.6	7.8	{	135.00
N-octyl hydrocupreidine hydrochloride	... { 1 in 28,000 1 in 29,000 (1/280)	3 3	2 2	7.7	7.8	{	140.00
Sec-octyl hydrocupreidine hydrochloride	... { 1 in 29,000 1 in 30,000 (1/290)	3 3	2 2	7.5	7.8	{	145.00

1+ indicates that it cannot be quickly decided whether the sperms should be graded as '1' or '2'. For explanation of symbols '0', '1', '2' and '3' see text.

TABLE II.

Effect of laboratory hydrocupreine derivatives on cary (guinea-pig) sperms.

Substance.	RESULTS.						Potency in terms of quinine hydrochloride which is taken as 1 unit.
	Strength of solution.	Control solution.			pH of test solution.	pH of control solution.	Spermicidal value, per cent.
		5 m.	30 m.	5 m. 30 m.			
Methyl hydrocupreine hydrochloride	...	3	3	2 0	7.6	7.8	1/50 25.00
	{ 1 in 5,000 (1/50) ... { 1 in 6,000 (1/60)	3	3	2 1	7.7	7.8	
Ethyl hydrocupreine hydrochloride	...	3	3	2 0	7.6	7.8	1/80 40.00
	{ 1 in 8,000 (1/80) ... { 1 in 9,000 (1/90)	3	3	2 1	7.6	7.8	
Iso-butyl hydrocupreine hydrochloride	...	3	3	1+ 0	7.7	7.8	1/160 80.00
	{ 1 in 16,000 (1/160) ... { 1 in 17,000 (1/170)	3	3	2 1	7.6	7.8	
Iso-amyl hydrocupreine hydrochloride	...	3	3	1+ 0	7.6	7.8	1/240 120.00
	{ 1 in 24,000 (1/240) ... { 1 in 25,000 (1/250)	3	3	2 1	7.5	7.8	

1+ indicates that it cannot be quickly decided whether the sperms should be graded as '1' or '2'. For explanation of symbols '0', '1', '2' and '3' see text.

EXPERIMENTS ON THE TRANSMISSION OF HUMAN SCHISTOSOMIASIS IN INDIA.

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THE question whether human schistosomiasis can be transmitted in India attracted attention after the war of 1914-18 when troops returned to India from Egypt, where they had contracted the disease. Though schistosomiasis has been reported in India from time to time since 1887, it has been mainly in persons who had visited endemic areas outside India, and only five cases have been recorded in the literature in persons who had never left India. The first case was reported by Powell (1903a, 1903b) in a Hindu resident of Bombay who had painless hæmaturia without any constitutional effects. Numerous ova with terminal spines were found in the urine. The second case was recorded by Sewell (1904) in a British soldier who had passed dark-coloured urine with numerous ova of *Schistosoma hæmatobium*. The patient had never served in any country except England and India, but it is not stated whether he passed through Africa on his way to India from England. The third case was reported by Hooton (1914) in a Parsi girl, aged 5 years, at Rajkot (Gujarat). She suffered from pain on micturition and passed smoky urine. Terminal spined ova were found in the urine. Eight years later another case was reported by Harkness (1922) in England in a soldier who had been in India for three years. He had been suffering from pain in the back and frequent micturition for two months with a history of a similar attack two years previously when stationed in India. During the second attack in England, ova of *S. hæmatobium* were found in his urine and the condition cleared up after treatment with antimony. There was no mention whether he passed through Africa on his way to and from England. The last case was reported from Rawalpindi by Andreasen and Suri (1945). This was in an Indian Sikh sepoy, a resident of the Ambala district in the Punjab; he had never left India. He was for a short time in Poona where West African troops were stationed at the time. Following a trivial injury in sports, he noticed blood in his urine and for two weeks suffered from dull pain in the lower abdomen and at the end of the penis after micturition. Large number of ova of *S. hæmatobium* were found in the urine every day. The ova disappeared after injections of tartar emetic.

It will be seen that between the 1914-18 war and the 1939-45 war, only one case of schistosomiasis, contracted in India, has been reported. Had a suitable molluscan host existed in this country, there would presumably have been many more cases.

In 1918, at the request of the Government of India, investigations on the transmission of the infection were undertaken by the Zoological Survey of India. Kemp and Gravely (1919) started their investigations in the neighbourhood of Hyderabad (Deccan), where some hundred men (returned from Egypt) suffering from schistosomiasis were then living. Fresh-water snails were collected from the locality and examined for cercariæ of *S. hæmatobium*, with negative results. Altogether 928 snails were examined and in 36 snails fork-tailed cercariæ were found, but these did not belong to the human schistosome group. Four hundred and fifty snails were exposed to active miracidia of *S. hæmatobium*; 251 were alive at the end of 4 to 8 weeks when they were dissected, but no parthenitæ were seen. This work was continued by Annandale and Sewell (1920). Annandale made an exhaustive survey of the fresh-water gastropod molluscs of India and Baluchistan excluding Assam and Burma. He found the aquatic molluscan fauna of the plains of India 'from Peshawar to Cape Comorin and from

Bombay to Calcutta ' extremely uniform. He laid down for the first time the geographical boundaries of the range of the different species and groups of snails. Species of the genus *Bulinus* (the chief carrier of *S. hæmatobium*) were not found within the limits of the Indian empire. In the same investigation Sewell repeated the experiments with living miracidia of *S. hæmatobium* on the more common freshwater snails of the Calcutta districts. He exposed 1,532 snails, of which 617 survived at the end of two months. They were then dissected, but no parthenitæ were seen. He also made an important study of the various forms of cercariæ in snails in India. He discovered a schistosome cercaria morphologically indistinguishable from that of *S. japonicum* in both *Indoplanorbis exustus* and *Limnæa acuminata*. This finding has, however, not been confirmed by other workers. Cullen (1924) found the ova of *S. japonicum* in the fæces of nine Chinese coolies in the border territory between Yunnan and the northern Shan States of Burma. The snail fauna of this locality was surveyed by Rao (1928). Every species of snail which was collected was examined, but *Oncomelania*, the carrier snail of *S. japonicum* in China, was not found. These snails were also examined for cercariæ, but no true schistosome cercaria was identified. No further cases of *S. japonicum* infection in man in that locality were reported between 1924 and 1928, although the immigration of the Chinese coolies from Yunnan into the northern Shan States had continued. No case of infection with *S. mansoni* either imported or indigenous has ever been reported in India.

THE PRESENT INVESTIGATIONS.

In May 1944, information was received that a large body of West African troops was stationed in the Ranchi area. Schistosomiasis is known to be endemic in West Africa, and it was expected that many of the troops would be infested with schistosomes. Examination of the urine and fæces of 22,317 African troops showed that 2,061 were passing ova of *S. hæmatobium* and 38 individuals the ova of *S. mansoni*. These examinations were carried out by military medical officers and the figures quoted have been taken from the report rendered to D.D.M.S., Eastern Command, by Major Rowland, R.A.M.C., Officer-in-Charge of the team. The presence of this large body of West African troops opened the question of the possibility of the spread of schistosomiasis in India, in spite of the fact that the carrier snails of schistosomes were not known to exist in India. However, in order to reduce the reservoir of infective material, all infested individuals were given adequate treatment for schistosomiasis. During this work the observation was made by one of us (S. N.) that in individuals with the history of hæmaturia or in individuals with very scanty ova in the urine, injection of antimony preparation often leads to the demonstration of ova in the urine or an increase in the number of ova in those cases where ova have previously been scanty. A survey of the freshwater gastropod molluscs around the area occupied by the troops was undertaken and transmission experiments were carried out with the snails which were collected.

TRANSMISSION EXPERIMENTS.

(a) With snails collected from the Ranchi area.

In June and July 1944, batches of snails were collected by one of us (S. N.) from the Ranchi area and from Chas (near Purulia). The snails were brought to Calcutta by railway and were kept in individual containers in fresh tank-water (from one of the local tanks of Calcutta). Ova of *S. hæmatobium* were concentrated by sedimentation of urine and were added to the water containing snails. In the first experiment the snails were all dead within four days. In the second experiment, instead of adding ova, a number of freshly hatched miracidia were added to each container. The total number of miracidia used was approximately 700. The mortality amongst the snails was very high. Except for five *Acrostoma* all the snails died within one month. These five *Acrostoma* were sacrificed at the end of two months and examination of these showed no infection. The species and the number of snails used in these two experiments were : *Indoplanorbis exustus* 29, *Acrostoma variabile* 7, *Limnæa luteola* 21, *Melanoides tuberculatus* 2, and *Vivipara bengalensis* 40.

Similar observations were made by Kemp and Gravely (*loc. cit.*) who found that the majority of snails brought from Hyderabad (Deccan) to Calcutta died during transit. It was

decided, therefore, to try transmission experiments with snails collected from the Calcutta area.

(b) *With snails collected from the Calcutta area.*

The snails were collected from the suburbs of Calcutta and were kept in large earthenware pots (bazaar quality) in tank-water containing water weeds. In order to prevent breeding of mosquito larvæ and also to prevent the snails from escaping, the pots were covered with coarse mosquito netting. There was no difficulty in maintaining a colony of snails. Laboratory-bred snails were obtained and transferred to new pots. Some of the snails caught in nature were found to be infected with single-tailed cercariæ; snails bred out in the laboratory were free from such infection. Snails used for transmission experiments were observed for a few days to make sure that they were free from infection and only those snails that did not pass any cercaria were used for transmission experiments.

Ova of *S. hæmatobium* were concentrated by repeated centrifugalization of the urine and the sediment was collected by a pipette and distributed on Petri-dishes. Tank-water which had been either filtered or boiled to eliminate cyclops and protozoa was added to the sediment and the number of ova and miracidia in each dish was counted, after which the dishes were filled with water. One snail was added to each Petri-dish and the snails were observed under the microscope to see if any penetration by the miracidia occurred. No such penetration was noticed in any instance. After about 18 hours of contact, the snails were removed to individual glass specimen-phials and kept in tank-water with a few water weeds. It was noticed that snails of the genus *Gyraulus*, *Limnæa* and *Vivipara* often tried to crawl out of the water and to prevent this, gauze cloth was tied round the top of the containers. The snails were kept in the laboratory and examined for from 8 to 14 weeks. The temperature in the laboratory during the period of observation, i.e. from June 1944 to May 1945, varied from 78°F. to 98°F. Many of the snails died within three days of exposure.

The racks of the phials containing the snails were exposed to sunlight for one hour and the phials were examined with a hand lens for cercariæ. This was done approximately three times a week. The water containing the snails was changed once a week. The snails that died were dissected and those that survived the period of 8 to 12 weeks were sacrificed. No parthenitæ were seen in any of these snails.

Table I shows the number of snails exposed and their mortality :—

TABLE I.

Snails.	Total number exposed.	Total number died.	Died 4-28 days.	Died over 28 days.	Sacrificed.
<i>Acrostoma variabile</i> ...	1	0	0	0	1
<i>Bithynia pulchella</i> ...	200	69	5	64	131
<i>Gyraulus convexiusculus</i> ...	159	125	19	106	34
<i>Indoplanorbis exustus</i> ...	59	51	10	41	8
<i>Limnæa luteola</i> ...	169	164	94	70	5
<i>Melanoides lineatus</i> ...	101	64	9	55	37
<i>Melanoides tuberculatus</i> ...	57	29	3	26	28
<i>Vivipara bengalensis</i> ...	142	67	12	55	75
TOTALS	888	569	152	417	319

Investigations on the transmission of *S. mansoni* through Indian snails had not been carried out before in India. Annandale and Sewell (*loc. cit.*) stated that they did not get a suitable case of *S. mansoni*. We were able to get a few infested African troops from whom a good supply of *S. mansoni* ova was available.

The ova were concentrated by repeated sedimentation of the fæces in water and from the sediment the ova were picked out with a fine pipette under the one-inch objective of the microscope. The ova were counted and added to the water in the Petri-dishes containing the snails. The ova took a longer time to hatch than those of *S. hæmatobium* but almost all the ova hatched in 24 hours. The snails were kept in contact with the miracidia for 18 hours and were then transferred to individual phials for further observation.

Some of these snails died within three days; these were discarded. The rest were dissected as they died or were sacrificed at the end of 8 to 12 weeks. No parthenitæ were seen in any snail. Table II shows the snails exposed and their mortality:—

TABLE II.

Snails.	Total number exposed.	Total number died.	Died 4-28 days.	Died over 28 days.	Sacrificed.
<i>Acrostoma variabile</i> ...	2	1	1	0	1
<i>Bithynia pulchella</i> ...	50	6	5	1	14
<i>Gyraulus convexiusculus</i> ...	36	20	5	15	16
<i>Indoplanorbis exustus</i> ...	59	43	32	11	16
<i>Limnæa luteola</i> ...	12	12	10	2	0
<i>Melanoides lineatus</i> ...	26	13	2	11	13
<i>Melanoides tuberculatus</i> ...	21	3	1	2	18
<i>Vivipara bengalensis</i> ...	33	8	4	4	25
TOTALS	239	106	60	46	133

Different species of *Planorbis* have been incriminated in different localities as intermediate hosts of *S. mansoni*; e.g. *P. boissyi* in Egypt and Abyssinia. *P. alexandrinus* and *P. herbeni* in the Sudan, *P. pfefferi* in Natal, *Australobis glabratus* (syn. *P. guadaloupensis*) in Venezuela and Porto Rico. Annandale and Sewell (*loc. cit.*) doubt whether true *Planorbis* occurs in India. The common species found in India have been brought under a new genus *Indoplanorbis* distinguished from *Planorbis* by anatomical differences in the soft parts.

It was thought possible, however, that *S. mansoni* might undergo development in this allied Indian species but our experiments with 59 of these snails gave negative results.

It may be mentioned that during the exposure the miracidia of both *S. hæmatobium* and *S. mansoni* were not attracted to any snail; they swam about without any attempt to penetrate the soft parts of these snails. Only in two instances was it observed that the miracidia of *S. hæmatobium* attached themselves to a *Melanoides* and an *Indoplanorbis* but after a short time they detached themselves again.

It was not possible to observe all the snails, but a few of each genus were observed under the microscope for as long as five hours at a stretch. No actual penetration was seen.

An extensive survey of the freshwater gastropod molluscs of India by various observers shows that the intermediate hosts of human schistosomiasis as reported from other countries do not apparently exist in India. India was exposed to this infection after the return of infected soldiers following the war of 1914-18. Although a few indigenous cases have been reported in individuals who had never left the country, no instance is known of any localized outbreak of the disease as one would expect if a suitable molluscan host was present. Against this it may be argued that the country was never exposed to such heavy infection as has been the case during this war of 1939-45; two thousand infected cases were present in one division alone. From the zoological point of view there is no reason to discredit the idea that the snails of India might not adapt themselves in future to human schistosomes. All the previous and present experimental work has been carried out in the laboratory. Under natural environmental conditions additional factors may be operative.

In the present state of our knowledge, it is not possible to explain the occurrence of the few sporadic cases of *S. haematobium* infestation in man in India which have been reported from time to time. Repeated examination of the local population for schistosomiasis in areas where African troops were located in India during the war of 1939-45 is indicated as a long-term policy. Transmission experiments and survey work were later carried out on an extensive scale by G.H.Q. (I.) Parasitology Research Team and a report by Major Hsu is under publication.

SUMMARY.

Attempts to transmit the infection of *Schistosoma haematobium* and *S. mansoni* through the common Indian snails were not successful.

Our thanks are due to the Indian Research Fund Association for a grant to carry out the present investigation, and also to Dr. B. Chopra, Director, Zoological Survey of India, for identifying some of the snails.

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A NOTE ON THE ACTION OF HEPARIN ON RUSSELL'S VIPER VENOM.

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THE following observations on the action of heparin on the venom of *Vipera russelli* (daboia) are sufficiently interesting to warrant publication.

Heparin* used in the experiments was a solution in physiological saline of the sodium salt of heparin of a strength of 10 mg. per ml., each mg. representing 110 Toronto units approximately.

The venom was a well-dried sample composed of a mixture of the venom extracted from several Russell's vipers. Its minimum lethal dose for pigeons and rabbits injected intravenously was 0.005 mg. and 0.15 mg. respectively. The sample may be considered as one of average toxicity.

TABLE I.

Action in vitro.

Daboia venom in mg.	Heparin in mg.	Sheep blood in ml.	Result.
0.10	Nil	1.0	Clot 45 sec.
0.01	"	1.0	Clot 1 min. 30 sec.
0.001	"	1.0	Clot 2 min.
0.0001	"	1.0	Clot 3 min. 30 sec.
Nil	"	1.0	Clot 8 min.
"	1.00	1.0	No clot.
"	0.10	1.0	No clot.
"	0.01	1.0	Clot after 24 hours.
0.10	1.00	1.0	No clot.
0.10	0.10	1.0	No clot.
0.10	0.05	1.0	Clot after 12 hours.
0.10	0.04	1.0	Clot after 8 hours.
0.10	0.03	1.0	Clot after 4 hours.
0.10	0.02	1.0	Clot after 1 hour.
0.10	0.01	1.0	Clot after 2 min. 40 sec.
0.10	0.005	1.0	Clot after 2 min. 5 sec.

* Heparin was obtained through the courtesy of Messrs. Eli Lilly & Co., to whom our thanks are due for their generous supply.

It will be seen from Table I that 0.10 mg. of heparin mixed with 0.1 mg. of daboia venom is capable of preventing *in vitro* the coagulation of 1 ml. of sheep blood.

In vivo experiments.

TABLE II.

Russell's viper venom and heparin mixed, incubated at 37°C. for 30 minutes and the mixture given intravenously.

Rabbit, weight in g.	Venom in mg.	Heparin in mg.	Result.
1,080	0.15	7.0	Survived.
1,400	0.15	6.0	„
1,935	0.15	4.0	„
1,870	0.15	2.0	„
1,590	0.15	1.0	„
1,730	0.15	0.5	„
1,525	1.50	10.0	„
Control {	1,910	Nil	Died, 8 min.
	1,810	„	Died, 4 min.

TABLE III.

Russell's viper venom and heparin mixed and the mixture injected intravenously immediately after preparation.

Pigeon, weight in g.	Venom in mg.	Heparin in mg.	Result.
310	1.0	5.0	Survived.
340	1.0	5.0	„
310	1.0	5.0	„
Control {	300	0.005	Died, 45 sec.
	320	0.005	Died, 1 min.
	360	0.005	Died, 45 sec.

TABLE IV.

*Russell's viper venom and heparin mixed, incubated at 37°C.
for 30 minutes and the mixture given intramuscularly.*

Rabbit, weight in g.	Venom in mg.	Heparin in mg.	Result.
1,675	10	20	Survived.
1,725	40	80	"
1,950	10	Nil	Died.
1,300	10	"	"

It is evident from the results reported in Tables II, III and IV that (1) heparin when mixed with daboia venom is capable of rendering inert the toxic fraction (or fractions) of the venom, (2) this action is as complete immediately after preparation as after incubation for half an hour, (3) 5 mg. of heparin can counteract the toxic effects of 1 mg. of venom representing 200 intravenous lethal doses for pigeons, and (4) 40 mg. of daboia venom which is a lethal dose for a human being does not cause death in a rabbit when this dose is given intramuscularly mixed with 80 mg. of heparin.

TABLE V.

*Heparin given intravenously followed by venom
intravenously 5 minutes later.*

Rabbit, weight in g.	Heparin in mg.	Venom in mg.	Result.	
1,275	5.0	0.15	Survived.	
1,555	5.0	0.30	"	
1,950	10.0	1.00	"	
1,755	20.0	5.00	"	
1,875	40.0	10.00	"	
1,650	40.0	20.00	Died, 4 hours.	
1,300	10.0	10.00	Survived.	
1,725	5.0	10.00	"	
1,760	2.5	10.00	Died, 2 hours.	
1,665	1.0	10.00	Died, 3 min.	
Control {	1,820	Nil	0.18	Died, 6 min.
	2,005	"	0.20	Died, 7 min.
	1,800	"	10.00	Died, 2 min.

The results in Table V show that (1) heparin can be given to rabbits intravenously without any untoward effects in doses up to 20 mg. per kilo, and (2) its action is as effective *in vivo* as *in vitro*, 10 mg. of venom representing over 60 lethal doses having failed to cause death in an animal given 5 mg. of heparin intravenously.

In the above experiment heparin was administered to animals before injection of venom, a condition quite different to what happens in an actual case of snake bite. This method had to be adopted as the effect of venom given intravenously is far too rapid for any subsequent attempts at treatment to be effective. —

The more natural line of approach would of course be the subcutaneous or intramuscular injection of venom followed by treatment with heparin. Unfortunately the absorption of Russell's viper venom by the subcutaneous or intramuscular route is so irregular and variable that it is difficult to draw accurate conclusions from such experiments. Anderson (1932) found the same difficulty and had to substitute the intravenous for the intramuscular route in the standardization of antivenene. In spite of these drawbacks it was, however, considered advisable to test the value of heparin under conditions approaching those obtained in actual practice. Venom was administered to rabbits by the intramuscular route and heparin intravenously as well as by local infiltration into the site of venom injection. The results recorded in Tables VI, VII and VIII despite a few anomalous findings, especially in untreated controls, are sufficiently encouraging to justify the therapeutic trial of heparin in definitely known cases of Russell's viper bite.

TABLE VI.

Russell's viper venom 10 mg. intramuscular ; heparin 5 mg. intravenous, repeated.

Rabbit, weight in g.	Details of heparin administration.	Total heparin in mg.	Result.
1,320	17×5 mg. during 48 hours after venom injection ; treatment commencing immediately.	85	Survived.
1,800	7×5 mg. in 24 hours ; treatment started 20 minutes after venom.	35	"
1,205	4×5 mg. in the first 80 minutes ; treatment started 20 minutes after venom.	20	Died, 12 hours.

TABLE VII.

Russell's viper venom 10 mg. intramuscular ; heparin intravenous and by local infiltration.

Rabbit, weight in g.	Local infiltration.	Intravenous.	Result.
1,700	10 mg. at once	5 mg. after 1, 5 and 9 hours.	Survived.
1,440	10 mg. at once	5 mg. after 30 minutes	"
1,550	15 mg. after 30 minutes	5 mg. after 30 minutes	"
1,680	15 mg. after 45 minutes	5 mg. after 45 minutes	"
1,550	10 mg. after 45 minutes	5 mg. after 45 minutes	"
1,735	Animal collapsed 29 minutes after venom. Then given 10 mg. intramuscular and 5 mg. intravenous.		Died, 4 hours, 8 minutes.
1,450 (venom 20 mg. intramuscular).	20 mg. at once	5 mg. after 10 minutes	Survived.

TABLE VIII.

Controls: *Russell's viper venom intramuscular, no treatment.*

Rabbit, weight in g.	Venom in mg. intramuscular.	Results.
975	10	Died, 42 minutes.
1,750	10	Survived.
1,950	10	Died, 11 minutes.
1,300	10	Died, 25 hours.
1,140	10	Died, 17 hours.
1,910	10	Died, 36 minutes.
1,745	20	Survived.
1,660	20	Died, 10 minutes.

DISCUSSION.

Experimental evidence presented in the paper shows that heparin is capable of counteracting the coagulant effect of daboia venom *in vitro*, 0.1 mg. of heparin when mixed with 0.1 mg. of venom preventing the coagulation of 1 ml. of sheep's blood for 24 hours. That it is also highly effective *in vivo* is indicated by the fact that a rabbit given 10 mg. of heparin intravenously followed 5 minutes later by 10 mg. of daboia venom (representing over 60 lethal doses) intravenously survives. It has also been shown that rabbits given a lethal dose of daboia venom intramuscularly and subsequently treated by administration of heparin can be saved. The exact mode of action of heparin in preventing death following daboia venom poisoning is not quite clear. Whether it acts by neutralizing the toxic fraction (or fractions) of this venom or merely by interfering with the coagulation mechanism of blood is under investigation.

Many substances such as potassium permanganate, gold chloride, etc., are capable of detoxifying daboia venom *in vitro* but unfortunately they are destructive to tissues and too toxic for parenteral administration. Heparin has the great advantage that it can be given intravenously and can render inert the toxic effects of daboia venom *in vitro* and *in vivo*. Evidence presented in this paper would suggest that it would be advisable to administer heparin intravenously as well as by local infiltration around the site of bite as early as possible. It is pointed out that a Russell's viper can give a maximum dose of 100 mg. to 120 mg. of venom in a full bite. A minimum lethal dose for a man is in the region of 40 mg. Theoretically even in extreme cases not more than 200 mg. of heparin will be required, while in most cases 100 mg. would probably suffice. However, the optimum dosage and methods of administration to be used in human cases can be arrived at only after extensive clinical trials preferably controlled by following the changes in the clotting time of the patient's blood. It is not suggested that heparin by itself can take the place of specific treatment. All that can be said for the present is that it is a useful remedy which has given most promising results in the treatment of Russell's viper venom poisoning in experimental animals and that it may be of value in human cases, particularly when specific antivenene is not available or

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